SAFE REUSE OF URINE

Screening of Stored Urine for Pharmaceuticals Using Chromatography

Cláudia Zambeze Kallio

Bachelor Thesis
December 2012
Degree programme in
Environmental Engineering

TAMPEREEN AMMATTIKORKEAKOULU
Tampere University of Applied Sciences

ABSTRACT

Tampereen ammattikorkeakoulu

Tampere University of Applied Sciences

Environmental Engineering

ZAMBEZE KALLIO, CLÁUDIA

Safe Reuse of Urine: Screening of Stored Urine for Pharmaceuticals Using Chromatography

Bachelor's Thesis,55 pages, Appendix 10 pages

December 2012

Urine has a large potential to be used as fertilizer due to the nutrient content in ionic form. As the amount of reused urine grows, the concern with the safety also grows when it comes to the presence of microorganisms and pharmaceuticals. In this paper, the concentrations of the nutrients, nitrogen, phosphorus, potassium, calcium and magnesium, were determined as well as the presence of pharmaceuticals and other chemicals. The determination nitrogen and phosphorus, was done by Hach-Lange DR 2800 spectrophotometer. The other nutrients were analyzed using the Flame Atomic Absorption Spectroscopy.

The presence of pharmaceuticals was done by High Performance Liquid Chromatography and Gas Chromatography-Mass Spectrometry. In the High Performance Liquid Chromatography, urine samples, were hydrolyzed with HCl. The organic layer was separated using a C18 SPE separation column. The organic layer was eluted with dichloromethane and then evaporated to dryness with a N_2-flow and recovered with MeCN prior to analysis in a 150 mm long, SB-C18 column.

In the Gas Chromatography-Mass Spectrometry method, urine samples were loaded in a C18 SPE column and eluted with methanol. The eluate was later dried using a nitrogen flow and dissolved in ultrapure water. After pH adjustment, the pharmaceuticals were removed with methyl-tert-butyl ether re-dried under a nitrogen flow and recovered with methanol prior to separation.

Concentrations in a g/L range were obtained for N,P and K, and for Mg and Ca in the concentrations were in mg/L range. Pharmaceuticals such as androsterone, epiandrosterone, prasterone 3-sulfate , 2,6-Di-tert-butyl-4-hydroxymethylphenol and caffeine were identified.

Keywords: urine reuse, chromatography, steroids, estrogens

FOREWORD

First of all I would like to thank my supervisor Dr Eeva-Liisa Viskari for supporting and encouraging my ideas and for trusting my judgement.

I would also like to thank the laboratory technicians Sanna Eljaala and Marjo Lehmusto for their patience and help during the laboratory sessions.

A very special thanks to Heli Knuutila for all the help with the measurements and methods and also for revising my thesis.

I would like also to express my gratitude to my colleagues with whom I have shared my time in the environmental laboratory discussing and talking, especially Alberto Freire López and Laura Hernandez Ruiz. Thank you for all the time we have spent trying to find the way into the unknown methods. I would have not done this well without you.

And last but not the least thank you to my family, Sami, Terttu, Samuli, Venla and Bruno for the patience, comprehension and support.

Tampere December 2012

Cláudia Zambeze Kallio

CONTENTS

ABBREVIATIONS

Ca	Calcium
CDC	Centers for Disease and Control and Prevention
E1	Estrone
E2	Estradiol
E3	Estriol
EE2	Ethinylestradiol
EU	European Union
FAAS	Flame Atomic Absorption Spectrophotometer
GC-MS	Gas Chromatography Mass Spectrometer
HPLC	High Pressure Liquid Chromatography
IARC	International Agency for Research on Cancer
I.D	Inside diameter
K	Potassium
LIC	Leptospirirosis Information Center
MeEE2	Mestranol
MeCN	Methane cyanide
MeOH	Methanol
Mg	Magnesium
N	Nitrogen
NPK	Nitrogen, Phosphorus, Potassium
P	Phosphorus
TAMK	Tampere University of Applied Sciences
TN	Total Nitrogen
TP	Total Phosphorus
WHO	World Health Organization

1 INTRODUCTION

During the past 50 years, nitrogen (N) based fertilizers have contributed to the increase of crops production. This increase was more significant in the industrialized than in the developing countries. Excessive use of nitrogen and phosphorus fertilizers has consequences to the environment. They can cause ground and fresh water contamination, health effects and destruction of the micro-flora. On the other hand, the lack of nitrogen in soils, can cause soil impoverishment (Knudsen et al., 2006, 3, 41).

The increase in the use of fertilizers did not contribute to the increase of the crop yield (Somani, 2007). One of the biggest problems with fertilizers is their poor efficiency. The efficiency of the fertilizers is from 20 to 50%. Once the fertilizers are applied to the soil, part of it is lost as ammonia, other part leaches to the ground in form of nitrates. In recent years there has been a lot of talk to improve the nitrogen efficiency. This efficiency is related to the amount of other macro and micro nutrients present in the fertilizers and their proper application (Mosier, Keith and Freney, 2007, 5-8).

Recently the price of fertilizers has gone up, increasing the food prices in many parts of the world. This problem could easily be solved if farmers had access to cheaper fertilizers with a great capacity to increase the yield of the crops. If urine is to be used as a fertilizer it would not only help food production, it would also provide proper sanitation for many people all around the world. In European Union (EU), urine is not acceptable as an organic fertilizer due to safety reasons, but if properly managed and treated urine would be a good solution specially to farmers who cannot afford fertilizers (Schönning, 2001,11).

Many organizations, such as the World Health Organization (WHO), understand the importance of urine reuse and for that reason they have created instructions on how to handle and use urine without spreading of infectious diseases.

2 OBJECTIVES

Urine reuse has been studied by the Degree Programme in Environmental Engineering of Tampere University of Applied Sciences for more than five years. The yield of urine irrigated crops and the concentration of nutrients have been studied as well as the presence of heavy metals and their possible implications to plant growth. The objective of this study was to determine the concentrations of the main nutrients used in plant growth and to determine the presence of pharmaceuticals in the urine, especially estrogenic hormones.

3 URINE REUSE

Urine is a fluid biopsy of the kidney and it provides a source of information about the human body condition. Urine is mostly water (95%) and the remaining 5 % are solutes such as ions and organic molecules produced in the body or ingested for example pharmaceuticals, food additives and preservatives just to mention some (Brunzel, 2004, 42)

Excreted urine contains nutrients and other substances which were used for different purposes. Over the time, urine has been used for religious, industrial, culinary, medical and cosmetic purposes. According to Steinfeld (2004, 10), urine had different uses in different parts of the world, conforming to local customs.

In Europe, urine was used as dye, in bread and cheese production, as a wound disinfectant and also was used to toast to someone's health. In other continents, urine was also used. For example in Africa and America, urine was used as medicine and to manufacture "urine bombs" (Steinfeld, 2004,10-21).

Human urine, along with animal urine, was also used in the production of salt, phosphorus, saltpetre and salt ammonia. From urine it is also possible to produce drinking water. Nowadays, urine is mostly used as fertilizers and in some places in urine therapy (Steinfeld, 2004, 21). Urine as fertilizer is mostly used in the east- Asia, in some African countries and in South America (Weckman, 2000, 5).

3.1 Urine as fertilizer

Every day, two million tonnes of waste waters are discharged in water. The waste arises from waste water treatment facilities, industrial and agricultural activities (Pacific Institute, 2010). In Finland, 20% of the total nitrogen in water is from human urine and 90% of nitrogen and 70% of phosphorus in waste water is from human excreta (Weckman, 2000, 3-4).

Separating and using urine as fertilizer would reduce the discharges of valuable nutrients to the environment, reducing the need of artificial fertilizers, and help to achieve the millennium development goals by improving sanitary conditions and

reducing hunger and malnourishment. The use of urine as fertilizer would also reduce the discharge of environmental contaminants such as pharmaceuticals, nitrogen, and phosphorus into the environment.

Despite all the potential urine has to be used as a fertilizer, there are concerns with the presence of microorganisms, pharmaceuticals and other chemical substances that we ingest and are excreted in the urine. Due to these factors, in order to use the urine as chemical, sanitary conditions need to be met.

The concentration of nutrients in excreted urine depends on the persons diet, water intake, type of nutrient, persons activity and climate conditions, table 1, (WHO, 2006,11).

Table 1: Excretion of nutrients from urine per capita in different countries

Country	Nitrogen kg/person year	Phosphorus kg/person year	Potassium kg/person year
China	3,50	0,40	1,30
Haiti	1,90	0,20	0,90
India	2,30	0,30	1,10
South Africa	3,0	0,30	1,20
Uganda	2,20	0,30	1,0
Sweden	4,0	0,37	0,90
Kenya	2,1	0,23	0,80

Source: Adapted from Jönsson and Vinnerås, 2004[1], Swedish EPA, 1995[2]; FAO, 2001[2]

[1] Cited in WHO, 2006,10
[2] Cited in Schönning, 2001, 7

In average, a person excretes 1,5 l of urine. This amount of urine can be used to fertilize a field of $1m^2$ depending on the type of crop. The efficiency of the urine uptake will depend on the soil conditions and application methods. Urine can be applied diluted or concentrated, depending on the amount of nutrients required by plants. It can be applied before sowing or in the initial plant growth stage. The best fertilizing effect from urine is obtained if the urine is incorporated into the soil, thus reducing the losses on ammonia (Rohde, Richert, Stintzing and Steineek, 2004; Morken, 1998 cited in WHO, 2006, 10-11).

3.2 Nutrients

Urine is a unique fertilizer in the sense that all the nutrients are in ionic form and ready to be used by plants. Nitrogen in urine is in the form of urea that is converted to ammonia and nitrates which are ready to be used by plants (Schönning, 2001, 8).

Nutrients are divided into macro and micronutrients. Macronutrients are the most required by plants, and they are nitrogen, phosphorus, potassium, calcium and magnesium. Micronutrients are sulphur, boron, zinc, iron and molybdenum. Even though they are taken in small quantities by plants they are important for the efficient uptake of all nutrients by plants (Akinyemi, 2007,103).

Nitrogen is the most important determinant of plant growth and crop yield. Plants lacking nitrogen have a small growth and yellowish leaves as nitrogen is also important in the photosynthesis. Phosphorus is considered the second most important macronutrient. Concentrations of phosphorus and potassium in fertilizers are important to increase the nitrogen efficiency. Calcium helps to maintain soil balance (pH) and improves water penetration and neutralizes cell acids. Magnesium plays the same role as calcium and is extremely important for chlorophyll molecules. The absence of magnesium affects the photosynthetic activities. (Akinyemi, 2007,103-111)

3.3 Microorganisms

Tortora et al. (1992) states that the urine of a health individual is sterile in the bladder, and excreted urine contains less than 10000 dermal bacteria per mL (according to Schönning, 2001, 11).

Pathogens present in urine are from faecal cross contamination and their survival in the environment can be affected by different factors (table 2). Sexually transmitted pathogens may be excreted in urine, but there is no evidence that their survival in the environment represents a health hazard (WHO,2006,34)

Urine is excreted between pH 4,8-7,5 and after the storage the pH raises up to 9. Most of the microorganisms excreted in urine will not survive pH values higher than nine due to the presence of ammonia (Schönning, 2001,7).

The most common pathogens found in human urine of an infected person are: *Lepthospira interrogans, Salmonella typhi and salmonella paratyphi; Schistosoma haematobium; Mycobacterium tuberculosis and Mycobacterium bovis; Mycospiridia; Escherichia coli and Cytomegalovirus.*

3.3.1 *Leptospira interrogans*

Leptospira interrogans, are bacteria that cause influenza like symptoms, and it is transmitted by urine of infected animals (Feachem et al., 1983; CDC 2003 according to WHO, 2006,34). Once the bacteria are excreted, it can survive in the environment months or years, if the survival conditions are met (moisture, neutral pH and temperatures of about 25°C). The bacteria enter the host through damaged skin, mucous membranes, lungs and conjunctiva membranes (CDC; 2003).

Leptospirosisis a occupational health hazard in developing countries and it is of importance in tropical and subtropical areas, with a 5-10% mortality rate (Schönning, 2004,3, LIC, 2011). Urine is not considered a source of contamination because of the low prevalence.

3.3.2 Salmonella typhi and salmonella paratyphi

Salmonella typhi and salmonella paratyphi are gram-negative bacteria responsible for the typhoid and paratyphoid fevers. The disease is transmitted by food and drinking water (Easmon, 2009). The bacteria are excreted in urine but the risk of transmission is low due to the rapid inactivation of the gram-negative bacteria (Höglund, 2001 according to Shönning, 2004,3).

3.3.3 Schistosoma haematobium

Schistossoma haematobium are parasitic blood, dwelling fluke worms endemic in Sub-Saharan Africa and Arabian Peninsula (IARC Monograps 100b, 4). It is one of the major human parasitic infections. The eggs hatch in fresh water, meaning that if there is no water available, the infectious cycle is broken, and this is what happens if urine is diverted from water (WHO, 2006,35; Schönning, 2001, 11)

3.3.4 Mycobacterium tuberculosis and Mycobacterium bovis

Mycobacterium bovis is responsible for the infection of tuberculosis in cattle. However, humans can be infected if they consume unpasteurized products from infected cattle (CDC, 2005). Tuberculosis infection spreads through air from one person to other. This happens when an infected person coughs, sneezes, speaks or sings. (CDC, 2005). Even though the bacteria are excreted in urine, the risk of contamination is low. Humans can transmit the bovine and human strain to cattle when there is human urine in the fields were cattle is fed (WHO, 2006, 35; CDC, 2012c).

3.3.5 Mycospiridia

Mycospiridia is a group of intercellular spore forming parasites. They can infect humans, fish, mosquitoes and others animals. Most cases of microsporidiosis are associated with HIV infection or other form of immunosuppression (Chiijide, 2012).

In humans the infection is obtained by inhalation of spores. These spores can be excreted in urine and faeces, and urine is a possible transmission route (Haas, Rose and Gerba, 1999;according to WHO, 2006, 35; CDC, 2012 b)

3.3.6 Cytomegalovirus

Cytomegalovirus, is a member of the herpes family. Is a common virus that infects people of all ages. In the United States alone the virus infects 50-80% of women by the time they are 40 years (CDC, 2010). The infection is transmitted through direct contact with body fluids (WHO, 2006, 35).

3.3.7 Escherichia coli

Escherichia coli, are a large group of bacteria that can cause diarrhoea, urinary tract infections, respiratory illness and pneumonia and other illnesses" (CDC, 2012a) Their presence in stored urine are suspected to be from faecal cross-contamination. *E.coli* is the most common pathogen in urine. However it does not present an environmental risk as its survival in the environment is short.

Pathogens can be transmitted trough urine but the risk of contamination is low. In order to reduce pathogen contamination in urine, faecal cross-contamination needs to be reduced. The inactivation of these pathogens in the environment will depend upon different conditions of temperature, pH, ammonia concentration, dilution of the urine and time. (WHO, 2006, 39, Schönning, 2001,13)

The conditions in which urine should be stored will depend on the valid monitoring and the risk management assessment (WHO, 2006, 70). The type of crops to be fertilized by urine will be defined by storage conditions (table 2). In households, urine can be used without any storage, if the crops are to be eaten four weeks after the urine application.

Table 2: Recommended crops for stored urine.

Storage temperature	Storage time	Possible pathogens in the urine mixture	Recommended crops
4°C	≥ 1 month	Viruses, protozoa	Food and fodder crops to be processed
4°C	≥ 6 months	viruses	Food crops to be processed and fodder crops
20°C	≥ 1 month	viruses	Food crops to be processed and fodder crops
20°C	≥ 6 months	Probably none	All crops

Source : Schönning, 2001, 26.

The urine mixtures considered in the table have a pH value equal or higher than 8,8 and the nitrogen concentration is 1 g/l. If the crops are to be consumed raw, the urine needs to be applied one month prior to harvest. This table does not represent the gram-positive spore forming bacteria (Shönning, 2001, 26).

3.3.8 Pathogen inactivation in diverted urine

Schönning (2001) has studied the effect of temperature, dilutions and pH on microbial inactivation. For each group of microorganisms, a representative species were chosen and the results were as follow :

- Bacteria: All bacteria belonging to Campylobacter and salmonella group were rapidly inactivated. Spore forming Clostridia were not reduced in 80 days. Low temperatures (5°C), neutral pH and dilutions kept the bacteria active for longer time.
- *Protozoa:* In urine control with pH 9, oocysts of *cryptosporidium parvum,* were inactivated better than samples with pH values of 5 and 7.
- Virus: The inactivation of the viral models, *rhesus rotavirus* and *salmonella typhimurium phage,* were inactivated after 180 days of storage at 5°C. At higher temperatures, 20°C, the inactivation happened between 35 and 71 days for both viruses.

4 PHARMACEUTICALS IN THE ENVIRONMENT

4.1 Pharmaceuticals

Pharmaceuticals are a large group of active chemicals with different chemical, physical and biological properties as well as different functionalities. They can be taken orally or intravenously, and they can be totally absorbed in the organism or be excreted in the original form or in the form of metabolites which can be more, less or as active as the original substances (Encyclopaedia Britannica, Krümmer, 2004, 3).

Pharmaceuticals in the environment come from discharges of contaminated waters from wastewater treatment plants, waste waters from pharmaceutical industries and hospitals, landfill leaching, animal farming and other sources (Adams, 2009,56; Krümmer, 2004, 4).

All over the world tonnes of pharmaceuticals are sold. Most of them are used but a large percentage is disposed unused. The unused pharmaceuticals are disposed because they have reached the due day or they were not entirely used by patients. Some pharmaceuticals are removed from the market due to their negative effects on human health as well as in the environment. Greinner and Rönnehahrt (2003), Sattelberg, (1999) cited in Krümmer,(2004,5), have reported that one third of the total volume of pharmaceuticals consumed in Germany and about one forth of these consumed in Austria respectively are disposed with the household waste or directly into the drain. In 2001 the consumption of pharmaceuticals in Germany was over 30,000 tonnes. A study, in Germany found that, 16% of the enquired people disposed solid pharmaceuticals via toilet and 44% disposed liquid pharmaceuticals via toilet (Keil, 2008, 7-8).

Animal production is other source of pharmaceuticals in the environment, (Boxall et al., 2003 according to Krümmer, 2004,5), manure containing pharmaceuticals is reused as fertilizer, thus allowing the pharmaceuticals to remain in soils and furthermore contaminate ground waters.

In the 1970's, pharmaceuticals such as hormones, heavy metals, polycyclic aromatic hydrocarbons, furans, pesticides and detergents, became of scientific interest. They were investigated to find their effects to the environment. Substances which are largely used,

ibuprofen, carbamazepine, fibrates and antibiotics, have very detailed analysis of their effects to the environment. In wastewater treatment plants, pharmaceuticals are removed by sorption, biodegradation, photo-degradation and hydrolysis (Krümmer, 2004, 6-7).

Pharmaceuticals are classified according to their restorative properties and sub classified according to their structures. The main groups of pharmaceuticals are listed in table 5 Antibiotics, cytostatics and hormonal compounds are of environmental concern due to their negative effects on the environment. Antibiotics and antimicrobials have the possibility to form resistance and may potentially disturb the environmental bacteria cycles. The concern with cytostatics is related to their carcinogenic, mutagenic and embrytoxic characteristics. Estrogens and hormonal compounds are of concern because their endocrine disruptor effects can be observed at very small concentrations (Adams, 2009, 57-59; Metcalfe et al., 2004, 67-71).

Table 3 Classification of pharmaceutical according to their therapeutic use

Type of pharmaceutical	Application	Examples
Antibiotics and antimicrobials	Used to treat bacteria caused infectious diseases and also to improve nutrient uptake in the gastrointestinal tract	Neomycin, ertapenem, cefalotin, teicoplanin, spectinimycin, amoxicillin, penicillin, levofloxacin, sulfonamide, tetracyclines and lincomycin
Analgesics and anti-inflammatory drugs	Used in pain relief and inflammation suppression in conditions such as arthritis, bursitis swelling and joint pain.	Codeine, methadone, morphine, fenoprophen, celebrex, oxaprozin diclophenac and ibuprofen
Anticonvulsants and antiepileptics	Used to control seizures	Dilatin, primidone, fluoxetine, and carbamazepine
Antihistamines	Used to stop histamine production	Cimetidine and ranitidine
Antidiabetics	Used to control blood sugars	metformin
Antipsicotics, antidepressants and anxiety drugs	used to treat psychotic and depression disorders.	Diazepam, buspirone and meprobamate
Beta-blokers	Used to treat cardiovascular problems (hypertension, arrhythmia and others)	Atenolol, celiprol and propranolol
Cytostatics	Used in cancer treatment. Some of these pharmaceuticals are.	Aclarubicin, belomycin, azathioprine, ifosfamide and cyclophosphamide
Estrogens and hormonal compounds	Used in birth control pills and in hormone replacement therapy.	Estriol, estrone, ethenylestradiol and, mestranol
Lipid-regulators	Used to control disorders such as hypercholesterolemia	Bezafibrate, gemfibrozil, clofibrate and fenofibrate
Stimulants	They may be narcotic or non narcotic. they may be used to stimulate the heart and respiratory system and relax muscles.	Caffeine, nicotine
X-ray contrast media	Used to allow x-ray visualization of structures within the human body	Diatrizoate, iomeprol andiopamidol.

Adapted from Metcalfe et al., 2004; Ikehata et al., 2006 (Citted in Adams, 2009, 57-59)

4.2 Estrogens in the environment: occurrence, effects and removal

Hormones are chemical messengers that carry signal between cells. Originally, hormones were classified in three different types, the peptide and protein hormones, steroid hormones and amino-acids related hormones. However, the constant discovery of new hormones, makes it impossible to classify them in only three groups. (Norman, Litwack, 1997, 2;Limpiyakorn, Homkling & Ong, 2009, 186).

Endocrine hormones are directly released into the blood stream and exocrine hormones are released into a duct before entering the blood stream or flow from cell to cell by diffusion. The largish endocrine glands are the pituitary, pineal, thymus, thyroid, adrenal glands and pancreas. Males produce steroid hormones in their testis and females in the ovaries and placenta(Limpiyakorn, Homkling & Ong, 2009, 186).

Natural steroids are produced by the adrenal cortex, testis, ovary and placenta in humans and animals. They are produced from chemical changes of cholesterol and are divided into six families see figure1 for the major pathways for the biosyntesis of steroid hormones (Norman, 1997, Mathews, Van Holde and Ahern, 2000; Ying, Kookana & Ru , 2002, 545; Limpiyakorn, Homkling & Ong, 2009 186):

- **Progestins,** which regulate the activities during the pregnancy, and are also the antecedents to all steroid hormones;
- **Glucocorticoids,** they promote the gluconeogenesis and pharmacological doses and suppress inflammation reactions;
- **Mineralocorticoids** regulate the ion balance by promoting the re-absorption of K^+ Cl^- and HCO_3^- in the liver;
- **D vitamin and its metabolites**;
- **Androgens,** which promote male sexual development and maintain male sexual characteristics;
- **Estrogens or sex hormones**, which support the female characteristics. They are responsible for maintaining the health of the reproductive

tissues, breasts, skin and brain. They are produced by males in the testis and by females in the ovaries and placenta. table 3.

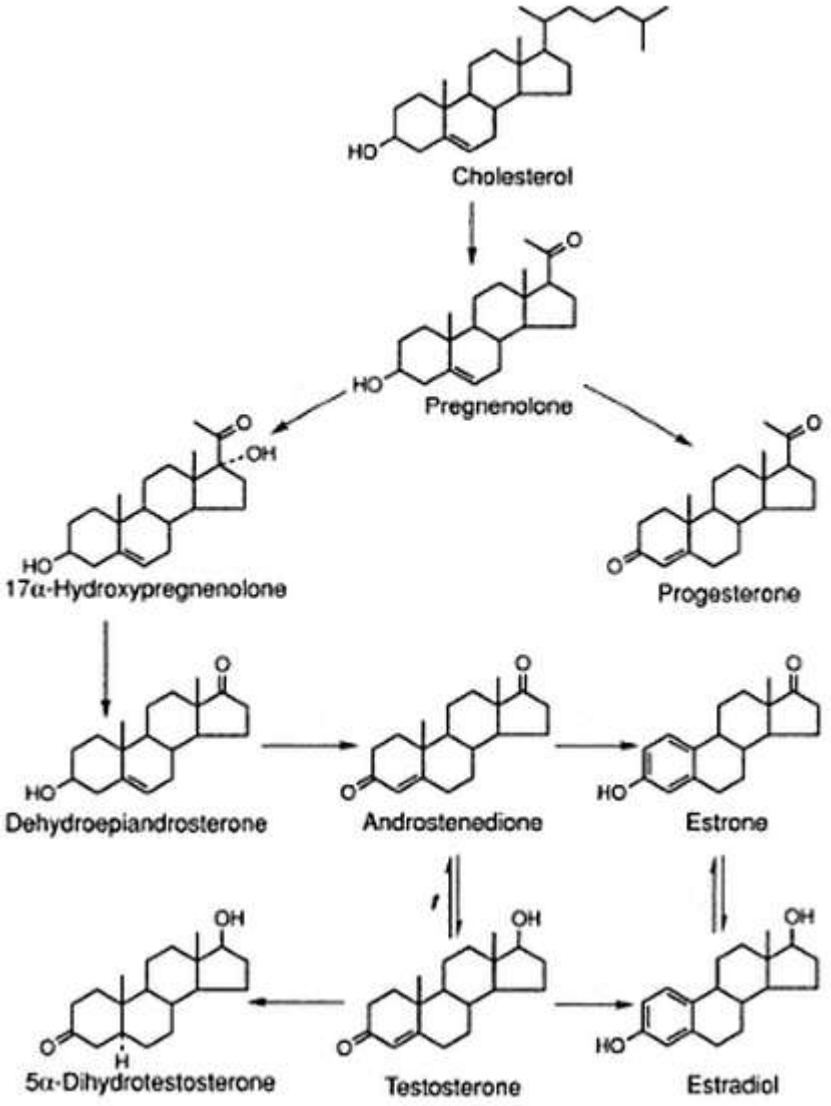


Figure 1: The main pathways for the synthesis of steroid hormones from Cholesterol (Zavod, 2008, 1304)

Table 6 Amount of hormones produced by mammals. Pregnant mammals can produce 1000 times more hormones than non pregnant(Brown, 2006).

Mammal	β-estradiol	Estrone	Estriol
Male Female Pregnant	1,5µg/24hr 0,3-5µg/24hr 26mg/24hr	35µg/24hr 2-205µg/24hr 375mg/24hr	 3-655µg/24hr
Milk cows Milk cows pregnant	170-12305µg/kg 1,35mg/kg	255-6405µg/kg 1,45mg/kg	
Bulls	<25µg/kg	<25µg/kg	
Pigs	2-645µg/kg	2-845µg/kg	
Horses		4005mg/kg	

Source : Shore and Shemesh, 2003[3].

Steroid estrogens are corticosteroids and sex hormones. The sex hormones are the androgens, estrogens, and progestagens. Some of the androgens are androgen, androstenedione, 20-hydroxyecdysone, testosterone and trestolone. Common estrogens are equol, equilin, estropipate , premarin, xenoestrogen, estradiol, estriol and β-estradiol, the last three ones being the active estrogens. Some of the Progestagens are drospirenone, dydrogesterone, progestin, hydroxiprogesterone (Tomšíková et al. 2012,2 Limpiyakorn, Homkling & Ong, 2009, 186-187).

Endogenous steroids estrone (E1), estradiol (E2) and estriol (E3) and exogenous steroids 17α-ethylnylestradiol (EE2) and mestranol (MeEE2) are substances of environmental

[3] Cited in Brown, 2006,

concern. The concern is due to the fact that they are endocrine disrupting chemicals (EDCs) (Tomšíková et al., 2012, 2; Ying, Kookana & Ru, 2002, 545-546) .

"Endocrine disruptors are exogenous substances that interfere with the endocrine system, and disrupt the physiologic function of the hormones in the body" (Miége et al, 2009). EDCs can be the naturally produced hormones, estriol, estrone and estradiol, synthetic hormones and other chemicals such as phthalates, 4-tert-penthylphenol, herbicides and other compounds (Adams, 2009, 59).

Steroid hormones are all 18-carbon molecules with four rings. They derive from the phenanthrene ring structure added to a pentano ring forming sterane ring. In figure 2 the main structure of steroids is represented, with the hexane groups named A, B, C, and pentane known as the D ring. The difference between different steroids are the functional groups in the positions C16 and C17 of the ring D (figure 3)(Norman, 1997, 51; Limpiyakorn, Homkling & Ong, 2009, 187).

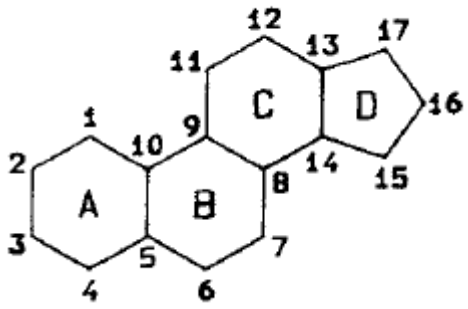


Figure 2: Ring structure of steroids. For estrone, estriol and estradiol, the OH group is located in the C3 position. (Norman, 1997, 51)

Estradiol is the most potent form of the female estrogens hormone, and it can be synthesised from estrone and testosterone. It represents 10 to 20% of the produced estrogens. It has a hydroxyl group on the C17 position that can point downwards to the plain forming the 17α-estradiol or upwards forming the 17β-estradiol. Estrone has a carbonyl group. It is 10 times less active than estradiol and it represents 60 to 80 % of the produced estrogens. About half of the body produced estrone comes from the ovaries while the other half comes from estradiol and the reconversion of estrone sulphate in the

adrenal gland. Estriol has an alcohol group on the position C16 and C17. It is the weak metabolite of estradiol. Ethinylestradiol is the first orally active synthetic steroidal hormone. It is an active ingredient for oral contraceptive pills production and in the hormone therapy (Norman, 1997, 51-56,65,66,72; Zavod, 2008, 1304, Limpiyakorn, Homkling & Ong, 2009, 187).

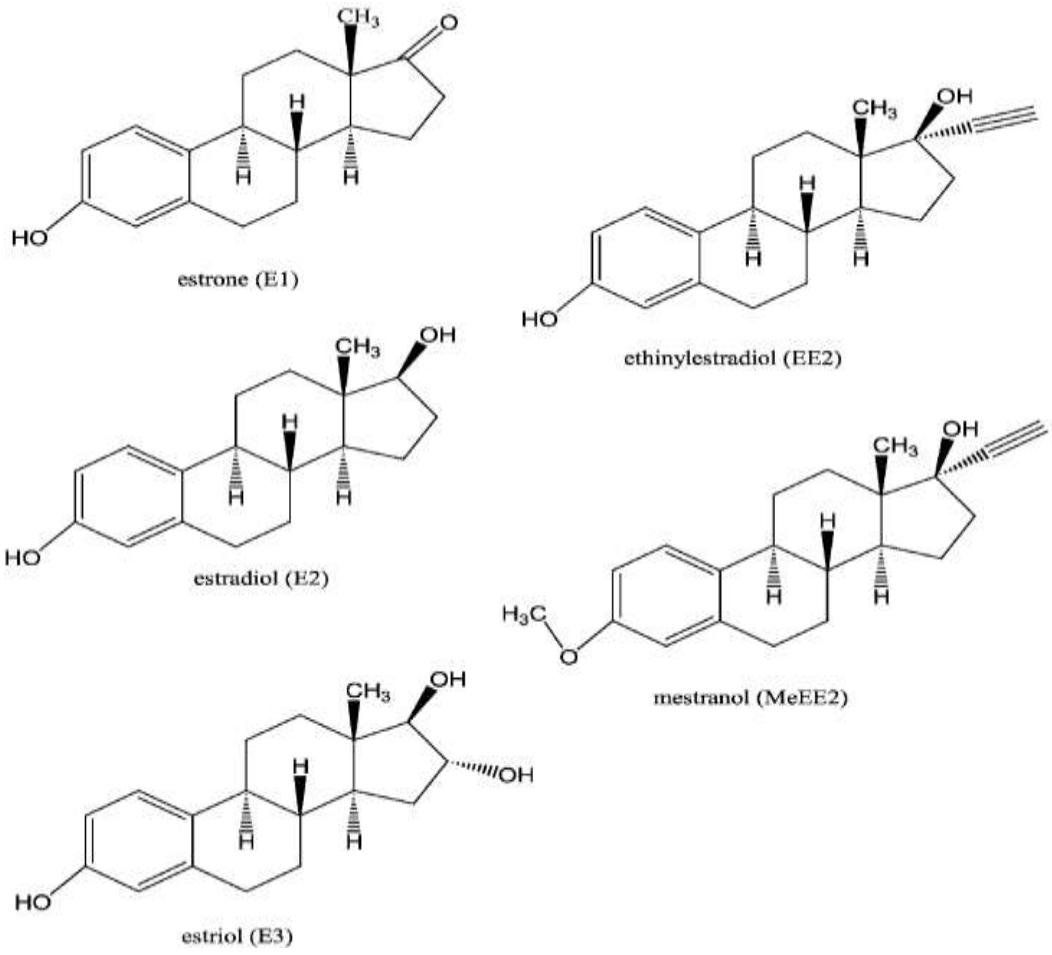


Figure 3: The structure of the main endogenous and exogenous hormones. (Ying, Kookana & Ru, 2002,546):

Estrogens have been found in water-run off, ground water, soil and in plants (Shore and Schemesh, 2003). Mammalian estrogens can also be isolated from entire plants. Up to 50% of the estrogens, in wastewater treatment plants are from pharmaceuticals (Shore and Schemesh, 2003). In surface water, estrogens come from water discharges from waste water treatment plants. In soils, the source of estrogens is suspected to be from, discharged sludge from waste treatment plants, poultry and cattle (Ying, Kookana & Ru, 2002,545).

Exposure of fresh water estuarine or fish living in the estuarine containing EDCs, may alter their sexual function and have toxicological effects in the ecosystems receiving high levels of estrogens (Tomšíková et al., 2012, 3).

Small concentrations of estrogens (ng/L) are potentially active in target tissues. Panter et al. (1997) and Gimeno et al. (1998), have studied the effects of endocrine disruptors in two different fish species, *Pimephales promelas* and *Cyprinus carpio* respectively.

Gimeno et al., applied 4-tert-penthylphenol, a potential EDC, and E2 during 90 days to male carp during spermatogenesis. The results showed that the exposure to these EDCs, caused the increase of vitellogenisis, a female lipoprotein, and the inhibition of spermatogenesis. Panter et al. have exposed minnous to different concentrations of natural estrogens during 21 days and obtained the same results as Gimeno. Concentrations of 1ng/l of estradiol have caused vitellogenesis in male trout.(Hensen et al., cited in Ying, Kookana & Ru. 2002, 546).

The effects of EDCs were also observed in plants. Alfalfa plants, (*Medigano sativa*) watered with estrogens containing water. The concentrations of estrogens varied from 10 to 137 ng/L. The irrigated plants, showed an increase of phytoestrogens one of the three major natural estrogens produced in plants (Shore et al., 1995).

Environmental effects of EE2 have been studied. Shulte-Oelmann et al. (1995, according to Shulte-Oelmann et al. 2004, 233-245) have studied the effects of EE2 to different snail species, and found that after exposure to different concentrations of EE2, the female snails inducted to the phenomenon called "superfemales". This meant that female snail had the formation of aditional female organs, gross malformations of the pallial oviduct section increasing the female mortality and massive stimulation of the oocyte and increasing mass production.

Different concentrations of estrogens have been detected in different countries. In waste treatment plants in Denmark, concentrations of 37ng/g and 49 ng/g dry weight of E1 and E2 have been observed (Andersen et al., 2005, 2).In Japan concentrations of 10,3ng/L of estrone and 4,8 ng/L of estradiol have been detected in surface water. In Arkansas mantled karsts aquifers, concentrations of six to 66 ng/L estriol and estrone have been

measured. Concentration of estriol have been identified only in Italy (Zhao et al.;2010, 2; Ying, Kookana & Ru, 2002,546).

In human urine, estrogens are excreted as inactive conjugates of sulphuric and glucuronic acids, which are soluble in water. In the liver, free estrogens undergo various transformations. They are oxidized to hydroxylated, deoxylated and methylated prior to final conjugation with glucuronic acid or sulphate. They can be later reconverted to active hormones by bacteria in the environment. 17β-estradiol rapidly oxidizes to estrone which is converted to estriol the major excretion product (Ying, Kookana & Ru, 2002,549; Lympiyakorn, Homklin & Ong, 2009, 194).

The presence of microorganisms in raw sewage binds the inactive estrogens leading to the discharge of active estrogens into the environment (Ying, Kookana & Ru, 2002,549). Microorganisms of English rivers were able to transform estradiol to estrone between 0,2 to 9 days when incubated at 20 °C and than estrone was further degraded at same rate (Jürgens et al., 2002, according to Ying, Kookana & Ru, 2002,550).

In waste treatment plants estrogens are removed by sorption and biodegradation fallowed by removal of excess sludge (Andersen, 2005,2). Due to their physiochemical characteristics, (Appendix 1) estrogens tend to de sorbed to solid phases. The sorption is directly related to the organic content and temperature. While high organic content of increases the sorption of estrogens, low temperatures will reduce it. Clara et al. (2004, according to Lympiyakorn, Homklin & Ong, 2009, 219-220), found that basic pH, 9, desorbed E2 and EE2. Sorption is suspected to delay the biodegradation process, because microorganisms have better access to organic matter when it is dissolved in water (Andersen, 2003,2005;).

In experimental studies, anaerobic batch with activated sludge, estradiol was oxidized to estrone and released without further transformation while EE2 was persistent (Ternes et al. 1999 according to Ying, Kookana & Ru. 2002, 550). In an experiment conducted by Layton et al. (2000 according to Ying, Kookana & Ru, 2002, 550), 70-80% of the added estradiol was mineralized to CO_2 within 24 hours.

Andersen et al (2003) found that E1 and E2 were degraded in the nitrification and dinitrification tanks, while EE2 was only degraded in the nitrification tank. In water and

sediment, the half-lives of steroids were estimated to be two to six days by Williams et al., table 4, (1999, according to Ying Kookana & Ru, 2002, 550).

Different microorganisms able to degrade estrogens have been found in activated sludge, soils , sediments and manure. In activated sludge *Rhodococcus* species, *Novosphingobium* species, *Mycobacterium, Sphingomonas, Escherichia, Fusarium proliferatum* are some of the microorganisms which were able to degrade E2, E1 and EE2 to metabolites or to compounds with no estrogenic activity (Lympiyakorn, 2009, 221-222).

Table 7 Half-life time for estrogens in river water

Estrogen	Half-life (days) in river water
Estrone	2-3
17 β-estradiol	0,2-9
Estriol	Not Reported
17α-ethynylestradiol	4-6

Source: Williams et al. (1999); Lai et al (2000) Jürgens et al. (2002) [4]

By comparing the influent and the effluent estrogens concentrations, Beronti et al. (2000 according to Ying, Kookana & Ru, 2002, 550), have calculated removal rates of 95 % for estriol, 87% for estradiol and 61 % for estrone. The low removal of estrone may be because of estriol transformation.

[4] According to Ying, Kookana & Ru (2002, 5)

Different rates of removal of estrogens have been observed in different countries. In Brazilian waste treatment plants removal rates of 64% to 83% for estrone have been observed and 92 to 99,9% of estradiol. In Japan, E2 removal rates in summer differ from the autumn values. In autumn the removal rates are over 99% when in summer from 7% to less than 99% (Nasu et al., 2000; Ternes at al., 1999 according to Ying, Kookana & Ru, 2002, 550).

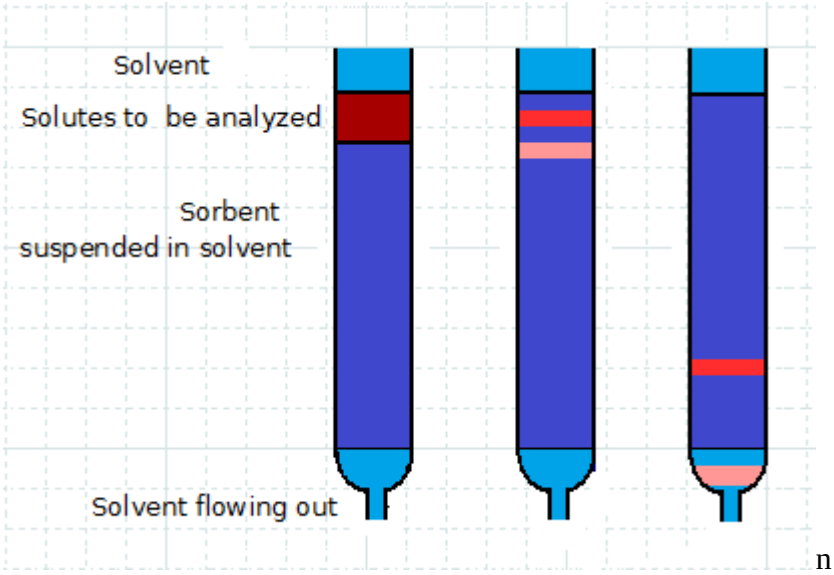
The persistence of estradiol and estriol in soils was studied by Colucci and Topp (2001) and Colucci et al. (2001). The studies showed the persistence of estrogens in soils will depend on the soils characteristics. The organic content of the soil, temperature and moisture content play an important role in the degradation of estrogens. After three days of incubation at 30°C, more than 56% of the applied estradiol was non-extractable. Estriol was abiotically transformed into estrone. The studies also showed that in the same treatment conditions, 17β-estradiol was removed two to seven times faster than 17α-ethynylestradiol.

5 EXPERIMENTAL SET UP

5.1.1 Chromatography

"Chromatography is a separation method were the analyte is allowed to interact with two physical distinct entities, the solvent (mobile phase) and the sorbent (stationary phase). The mobile phase is liquid or gas, and it moves the sample components through a region containing solid or viscous liquid stationary phase" (Boyer, 2006, 116 Harris, 2007,506). The figure 4 illustrates the principle of chromatography.

The sample entering the column is called the eluent and the one leaving is called the eluate. The time needed for a solute to be eluted from a chromatography column is the retention time. The results from the detection are displayed in the chromatogram as function of elution time (Harris, 2007,507).



n

Figure 4 Principle of chromatography. once the sample is injected the solutes separate (ii). The solute with affinity with the stationary phase will stay longer in the column (iii). (Modified from Harris, 2007, 506)

There are different types of chromatography such as adsorption chromatography, partition chromatography, ion exchange chromatography, molecular exclusion chromatography and affinity chromatography. They are categorized according to interaction between the solute and the stationary phase. This paper will refer only to partition chromatography because the analysis were done by partition chromatography.

In partition chromatography, a solid stationary phase and a liquid or gaseous mobile phase are used. The substances which are more soluble in the mobile phase will stay shorter in the column and the ones with more affinity with the solid phase will stay longer (Harris, 2007, 508; Dean et. al., 2002, 204).

Gas Chromatography (GC) involves the injection of a small amount of gaseous sample into moving stream of gas (carrier gas nitrogen (N_2), hydrogen (H_2)or helium (He)) through a hot column packed with solid particles and impregnated with a non volatile liquid (figure 5). The components of the sample move in the column at different rates thus reaching the detector at different times. Once the solute reaches the detector the results are presented in a chromatogram. In GC helium is the most common carrier gas and it is compatible with most detectors. The temperature of the column needs to be raised gradually during the separation not only to reduce the retention time and increase the pressure, but also to allow better detection (Harris, 2007528-532;Sawyer at al. 1984, 321-322).

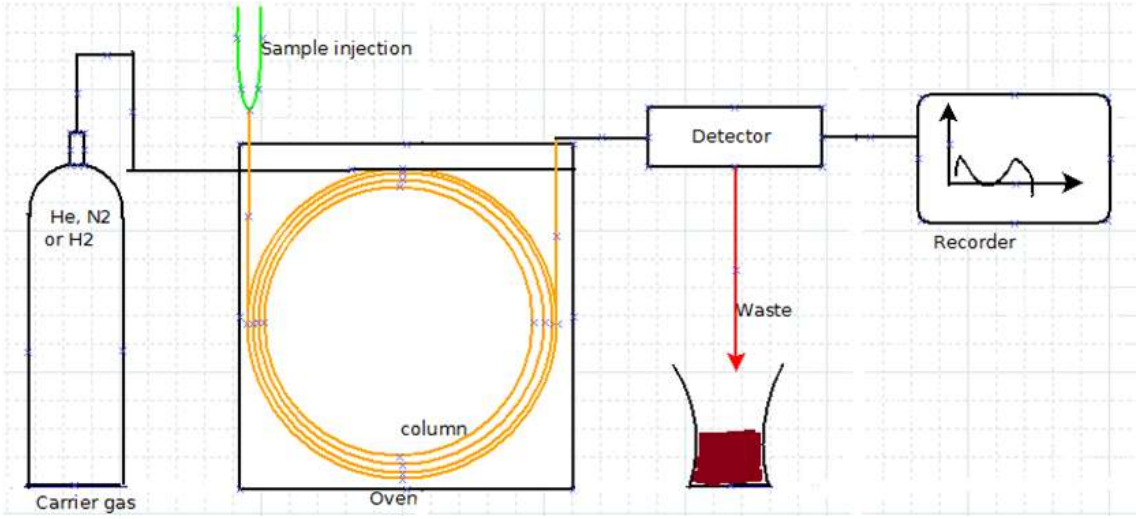


Figure 5 Main components of a GC system (Modified from :Dean et al.,2002, 211)

Mass Spectrometry (MS), is a technique that measures the masses of atoms or molecules or fragments of molecule. It does so by comparing the mass to charge ratio (r/z) of a gas phase of ions and provides a measure of abundance of each ionic species. Mass Spectrometers operate by braking up gas phase ions in a low pressure environment by interaction of magnetic or electrical fields on the charged particles" (Harris, 2007, 478; Kitson, Larsen and McEwen, 1996, 9).

GC-MS, is a combination of GC to the MS. Where the GC separates the components in time in gaseous form and the MS aids the structural identification (Kitson, Larsen and McEwen, 1996,3).

The GC-MS is an important analytical tool as it is cheaper than other methods, and it also has library of spectral database which can be used to compare the masses and the intensities of the fragments. The limiting characteristic with GC-MS is the fact samples need to be volatile. In figure 6 there is an example of a GC-MS system (Mcmaster & Mcmaster, 1998, 5-7; Kitson, Larsen &McEwen, 1996,3).

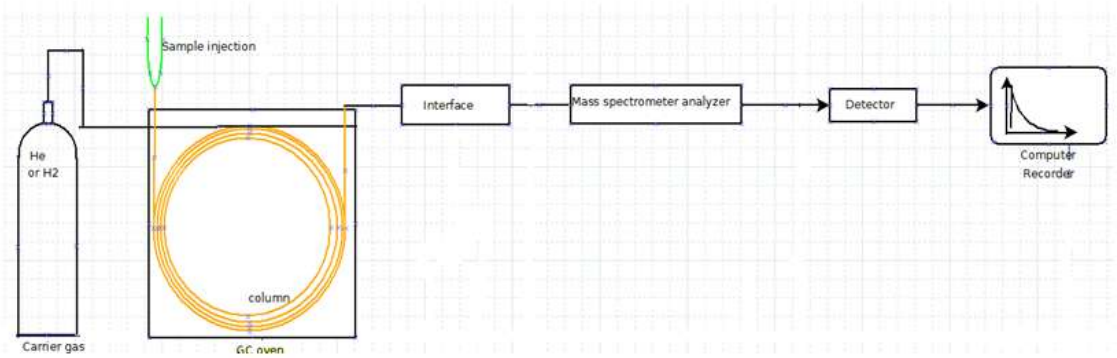


Figure 6 GC-MS system diagram (Modified from :McMaster &McMaster, 1996,7)

High Pressure Liquid Chromatography (HPLC) is a separation method that uses high pressure to force a solvent trough closed columns containing very fine particles that give high resolution separations. The biggest advantage of HPLC over GC-MS is the fact that samples do not have to be volatile. Other advantages of HPLC are the resolution speed compared to other methods, the columns do not need repacking or regeneration, the parameters can be easily adjusted and the analysis can be easily automated, the samples can be in small or large sizes (Harris, 2007, 556 .McMaster, 2007, 3-8)

The HPLC consists of a container with the mobile phase, a pump, a sample injecting valve that can be from 10 to 500µL, a column where is the stationary phase, an UV detector and a recorder (figure 7). The HPLC comes with a oven to heat the columns thus reducing the retention times and improving the resolution (Harris, 2007, 556-557).

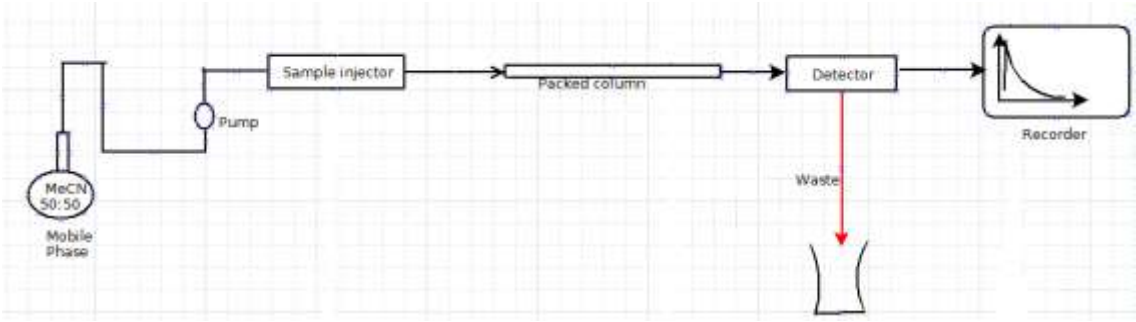


Figure 7 An HPLC system diagram (Modified from McMaster, 2007,7)

The compounds in the column are identified based on their retention times by comparing the unknown solution retention time to a known standard solution retention time. Before choosing the type of coated column to be used it is important to know the characteristics of the particles to be analyzed. These characteristics include the size, solubility, polarity and so on. Steroid hormones for example are nonpolar, so they require polar conditions and separate better C18 silica coated column. HPLC columns are expensive, so is better to used HPLC grade solvents and also to filtrate the samples and chemicals prepared from solid salts. Heating the columns improves the analysis, but it also can degrade the stationary phase and reduce the columns life time. The most common stationary phases in HPLC are the highly pure spherical microporous particles of silica which are permeable to solvent and have a surface area of several hundred square meters. The recommended pH for silica columns is between 2,5 to 7,5. pH values below 2 may remove the bonded phases and pH values over 8 may dissolve the column. (Harris, 2007, 556-568; McMaster, 2007,23-27)

5.1.2 Equipments and Materials

For the total nitrogen (TN) measurements, LANGGE LCK 283TN kits were obtained from Hyxo Oy Finland. The HT200S thermostat was used to aid the digestion and the samples were analyzed using Hach-Lange DR 2800 Spectrophotometer.

For the total phosphorus (TP), samples were analysed by the Hach-Lange DR 2800 Spectrophotometer program 490, reactive phosphorus. The 5,25 N H_2SO_4 solution was prepared from the 98% concentrated H_2SO_4 and the 5 N NaOH was prepared from the 50% NaOH solution. The glass wear was rinsed with a 1 M HCl solution to remove impurities.

The pH measurements were done using the Mettler Toledo FE 20 FiveEasy meter. The meter was calibrated using calibration standards pH 4,0 and pH 7,0.

For the chromatographic analysis, pure steroids were purchased from Sigma-Aldrich. Estrone≥ 99%, 17β- estradiol ≥ 98%, estriol ≥97%. The Toxi-tube A for organic layer separation was obtained from Agilent technologies. The samples were analysed by the Agilent 1100 series HPLC system and the 6890N network GC system and the 5973 Network Mass selective detector for HPLC and GC-MS respectively.

6 ANALYSIS METHODS

Urine analysis was divided in two parts: nutrient and pH measurements and urine screening for identification pharmaceuticals in particular steroid hormones.

6.1 Analysis of total nitrogen

TN was determined using the Hach-Lange DR 2800 Spectrophotometer. The samples were treated according to the LANGE LCK 283 Total Nitrogen kit instructions, figure 8. All the chemicals, the reaction tubes and the cuvette tests for the analysis came with the kit. The different chemicals and tablets were named A, B, C and D.

Figure 8 LANGE LCK 283 Total Nitrogen kit (Freire López, 2011, 42)

The principle of the kit is that the nitrogen from the sample will be oxidized to nitrate by peroxide sulphate. Once the nitrogen is converted to nitrate, it will react with 2,6-dimethyl phenol in a solution containing sulphuric and phosphoric acids.

500 µl of diluted urine samples was transferred to a reaction tube followed by 2,0 mL of sodium hydroxide (A) and one oxidant tablet (B). The tube was covered and placed in the HT200S thermostat (figure 9) for 15 minutes in the HT program (175°C). Once the

samples were digested and at room temperature, one micro cap (C) was added and the tube inverted few times to make sure that all the chemical was removed from the cap. 500 µL of this sample was transferred to a cuvette test. 200µL of the solution D was transferred to the same cuvette test and the cuvette was inverted few times and the sample was left to react for 15 minutes. After that the cuvette was placed in the Hach-lange DR 2800 for TN readings.

Figure 9 HT200S thermostat

6.2 Analysis of total phosphorus

Total phosphorus from urine was measured with Hach-Lange DR 2800 Spectrophotometer, figure 10. First the glass wear was rinsed with HCl:water 1:1. The samples were diluted 200 times in order to be in the detection range. 25 mL of the sample were transferred to a 125 mL Erlenmeyer flask, and mixed with one persulfate pillow powder. To this mixture, 2ml 5,25 N H_2SO_4 was added using a 1 mL calibrated pipette. The sample was gentle digested on a hot plate for 30 minutes, making sure that the volume inside the flask was never less than 20 mL by adding distilled water.

Figure 10 Hach-Lange DR 2800 (Freire López, 2011, 41)

The sample was then left to cool at room temperature. Once the sample was cold, 2 mL 5 N NaOH was added to the flask using a 1 mL calibrated pipette. The sample was then transferred to a 25 mL graduated cylinder and filled to the mark with distilled water. The sample was then analyzed with the Hach according to the instructions. The results measured from the Hach are in mg/l of phosphate ion. The volume of added water was carefully controlled as it should not exceed 20 mL.

6.3 Analysis of potassium, magnesium and calcium

Potassium, Magnesium and Calcium nutrients were determined usin the Perkin Elmer A Analyst 400 Atomic Absorption Spectrometer, figure 11. First the samples were filtrated with a Whatman ashless filter paper 589/3 with retention capacity less than 2 µL. The calibration solutions were prepared from 1000 g/l standard solution of the nutrient to be analyzed. The concentrations for the standards were determined by the chosen wavelength for the measurement.

Figure 11 Perkin Elmer A Analyst 400 Atomic Absorption Spectrometer

Once the samples were filtrated, they were diluted in range of the calibration solutions. For K 10 mL of urine was used and the calibration solutions prepared where of 30, 60, 90, 120 and 150 mg/L. For Ca, 50 mL of urine were used and calibration solutions of 1, 2, 3 and 4 mg/L were used. For Mg 10 mL and 15 mL of urine were used, and for the calibration curve 50, 100, 150 and 200 µg/L solutions were used.

The samples and standards were placed in 100mL volumetric flasks, were 1 mL 10% $LaCl_2$ was added and the flasks were filled to the mark with a 0,2 % HNO_3 solution. The HNO_3 and $LaCl_2$ solutions were added to remove interferences and to prevent the signal to be depressed.

6.4 pH Measurements

The pH was measured twice using the Mettler Toledo FE 20 FiveEasy, figure 12. The first measurement immediately after collecting the samples from the basement storage, and after one week of storage in the fridge. For this measurement, 100 mL of sample was placed into 150 mL beaker, and stirred in a magnetic stirrer plate for five minutes to mix all the particles in the urine. Before any measurement the pH meter was calibrated in accordance with the instructions.

Figure 12 pH meter Mettler Toledo (Freire López, 2011,41)

6.5 Pharmaceuticals determination

The screening of urine for pharmaceuticals detection was done by High Performance Liquid Chromatography and Gas Chromatography Mass Spectrometry.

6.5.1 HPLC method 1

Adapted from Lunn (2000), in this method, 1mL of urine and 3 mL of water was added to the Toxi-tube A and gently mixed by inversion for five minutes and centrifuged for five minutes at 1500 rpm. The organic layer was removed and gently dried to dryness under a nitrogen stream. The sample was reconstituted with 50 µL MeCN: water v/v 50:50, and gently mixed for 10 s and centrifuged for two minutes at 7500 rpm. The sample was filtered with a plastic cartridges, 0,54 µL, and 10 µL of the sample was injected into the HPLC.

6.5.2 HPLC method 2

Used by Mao et al., (2004), 500 µL of urine were hydrolyzed in the presence of methanol (500 µL) and HCl (50 µL 37%). The sample was left in the oven at 50°C for one hour. After one hour, the pH was adjusted to 3 with a 5 M NaOH solution and the solid phase was separated using the IST C18/SO_3^- 100 mg/mL HCX-3, figure 13. The solid phase separation column (SPE C18) was conditioned with water (4 mL) and methanol (4 mL) sequentially. The sample was passed through the column and eluted with 8 mL dichloromethane. The eluent was gently dried under a nitrogen stream and recovered with 1mL MeCN:water 50:50 and the sample was ready to be analysed. For both HPLC methods, different condition were tried to achieve the best results. The Agilent 1100 series HPLC system, figure 14, was used for the measurements. The used column was a 150 mm Agilent Zorbax Rapid C18.

Figure 13 separation of the organic layer using SPE column

Figure 14 Agilent 1100 series HPLC system

Three different mobile phases were used in different combinations. Mobile phase A MeCN: Water v/v 50:50, mobile phase B MeOH: Water v/v 60:40, mobile phase C 50 mM Na_2HPO_4 buffer. The buffer was prepared by diluting 6,15g of NaH_2PO_4 into 500 mL pure water and adjust the pH with 37% HCl. The mobile phases combinations where: A/D 50:50; B/C 50:50 and A only.

The column temperature was 30°C and different injection volumes were tried 10, 20 and 50 µL. Three different wave lengths were also tried 200, 201 and 210 nm.

6.5.3 GC-MS method

This method was used by Zhang et al. (2009). The first step in this method was to prepare 1 mg/mL stock solutions from the standards E1, E2 and E3. The stock solutions were prepared by dissolving the standards in methanol. The working solutions of 40 µg/mL were prepared by diluting the stock solution in acetone.

These samples were stored at 4°C and the volumetric flasks were covered with aluminium foil. The SPE column, HCX-3 IST C18/SO_3^- 100 mg/mL, was activated with ultrapure water and methanol 4 mL of each. 5 mL of urine was passed through the separation column. Once all the urine went through the column was rinsed with 5 mL water to remove impurities and eluted with 5 ml methanol. The eluate was dried with nitrogen flow and restored with 1mL ultrapure water. The pH was raised by adding 100 mg of solid buffer ($NaHCO_3$:Na_2CO_3 w/w 8:1; pH 8,8-8,9).

Once the pH was adjusted, 5 mL methyl-tert-butyl ether was added and the sample was vigorously shaken for 1 minute and centrifuged for 15 minutes at 4000 rpm. The upper layer was than evaporated to dryness with a nitrogen flow. The residue was dissolved in 100 µL methanol for the GC-MS analysis.

The separation was performed by the 6890N network GC system and the 5973 Network Mass selective detector both from Agilent technologies, figure 15. The used column was a capillary column (of 30 m length, x 0,25 mm I.D x 0,25 µm film thickness).

Figure 15 6890N network GC and the 5973 Network Mass detector

The column flow was 1,0 ml/min, the carrier gas was helium. The injection was in splitless mode at 260°C. The transfer line temperature was set at 300°C. The MS analyzer was set at 70eV, electron impact source temperature of 230°C and solvent delay was 1 minute.

The initial oven temperature was 100°C (5 minutes) raised up to 250°C with a ramp rate of 25°C per minute. The temperature was kept at 250°C for 7 minutes and then raised to 310°C at ramp rate of 30°C per minute. The identification of the pharmaceuticals was based on the GC-MS spectral database.

7 RESULTS

7.1 Nutrients and pH

The results from the nutrient measurements can be seen on the table 3, concentrations are in mg/l and the NPK ratio is 11:1:10. The pH measurements are on the table 6. The difference between both measurements is very small and the pH values are high considering that the urine is already diluted.

Table 8: Concentrations of the measured nutrients

Nutrient	Concentration mg/l
Nitrogen (total)	1440
Phosphorus (Total)	(390 PO_4^{-3}) 130
Potassium	1330
Magnesium	0, 76
Calcium	2,5

Table 7: pH results from stored urine immediately after collecting it from the basement storage and before nutrient measurements

Temperature °C	pH values
8,5	8,96
22	9,06

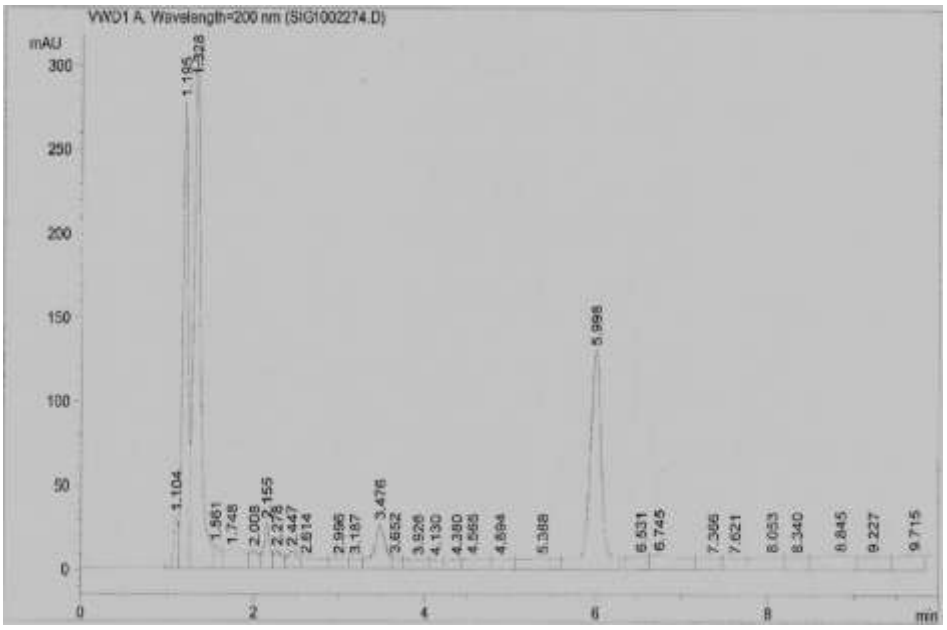
7.2 Chromatography results

The results obtained from chromatography are organized by the type of chromatography used in the separation process.

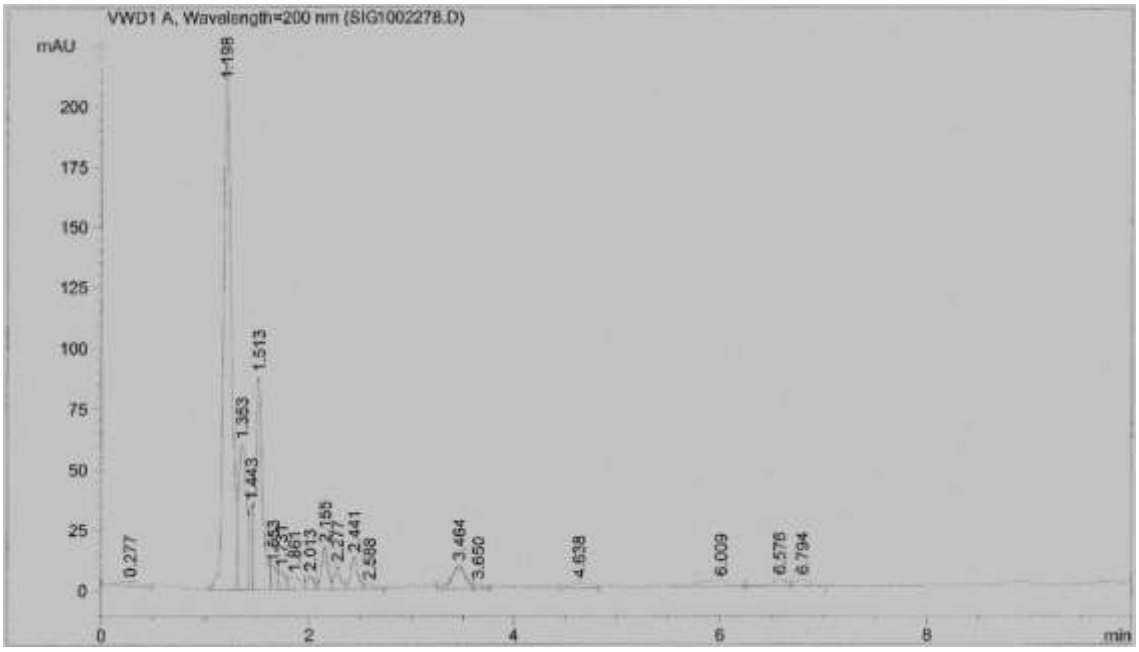
7.2.1 HPLC results

Two different methods were used. Below, are the chromatograms for the standard estrone 100 mg/l and for the sample. Different injection volumes, wavelengths and different mobile phases were tried with different results. The best results were obtained with the second method using 200 nm wave length, 20 µL injection volume and mobile phase A/C v/v 50:50 and retention time 6 minutes. The results below show the chromatogram for the standard solution of E1 (100 mg/L), chromatogram1, the chromatogram 2 represents results from the urine from one individual in the hormone replacement therapy (1 mg estradiol/2,5 mg progesterone) and chromatogram 3 the results of stored urine from the urine diverting toilets.

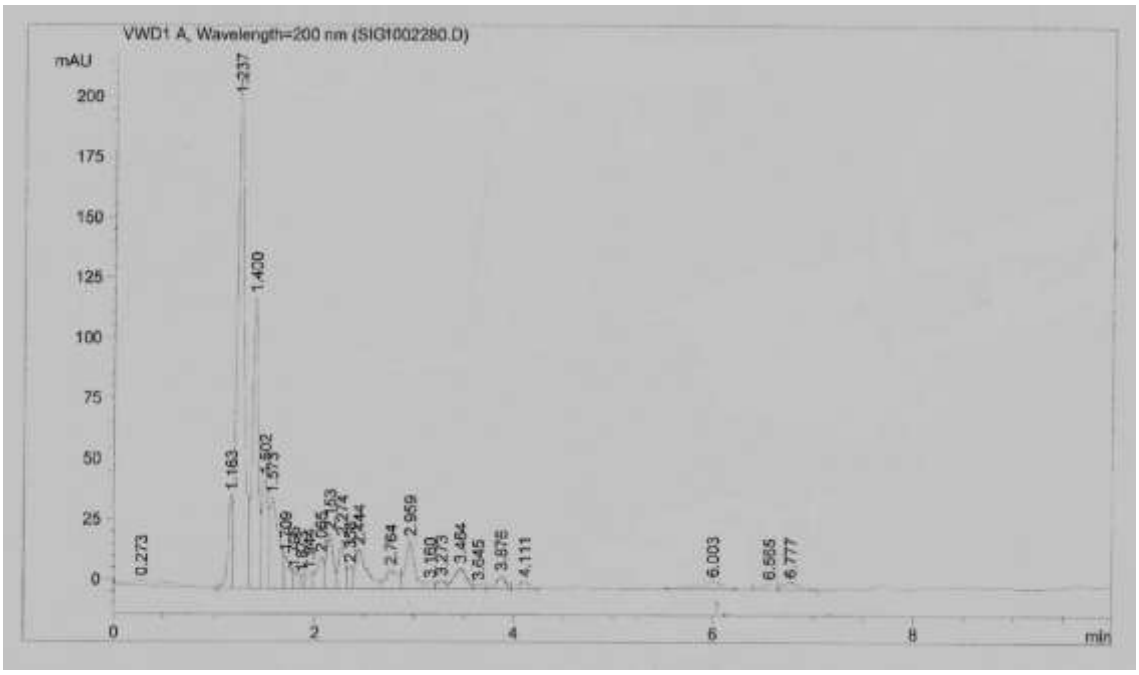
From the chromatograms can be seen that the urine from one individual contains less substances than the urine from several individuals. The peaks are not well defined but that could be achieved by spiking the samples and also by using gradient conditions. In this experiment isocratic elution was used. Chromatograms for method 1 are in appendix1.



Chromatogram 1: Chromatogram for the standard estrone solution (100mg/l) with retention time 5,998 min, wavelength 200nm



Chromatogram 2: Chromatogram for the hormone replacement urine with retention time 6,009 minutes. There is interference in the retention time with other substances**.**

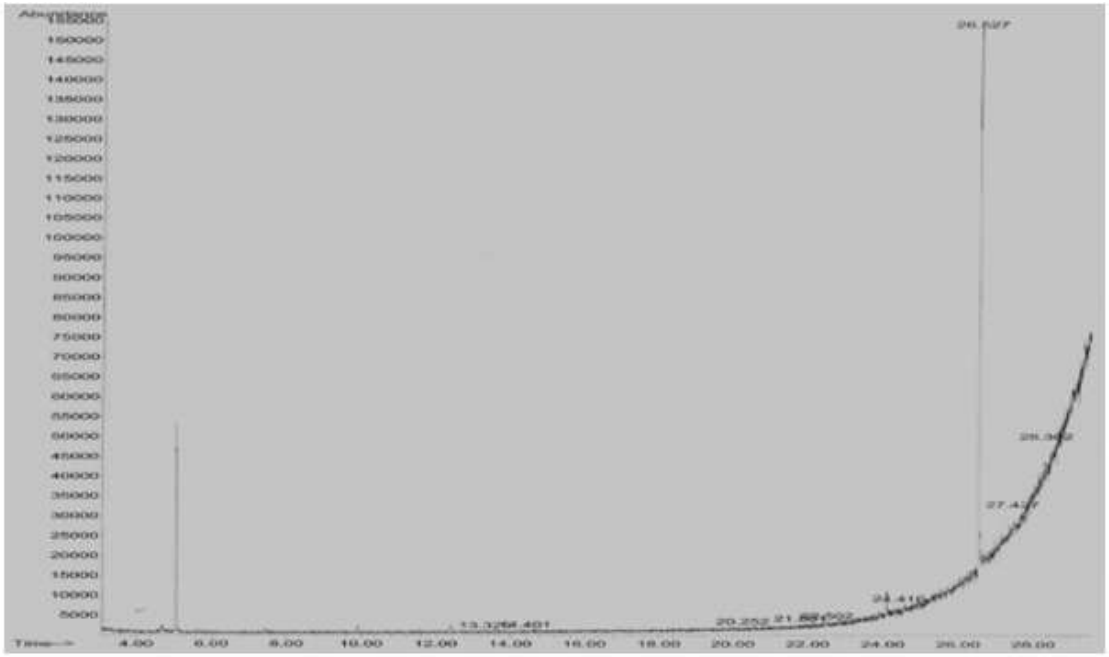


Chromatogram 3: Chromatogram for stored urine with retention time 6,003 minutes. It is clear that there are numerous substances in this samples causing interference with hormones. The peak is better defined that the peak from chromatogram 2.

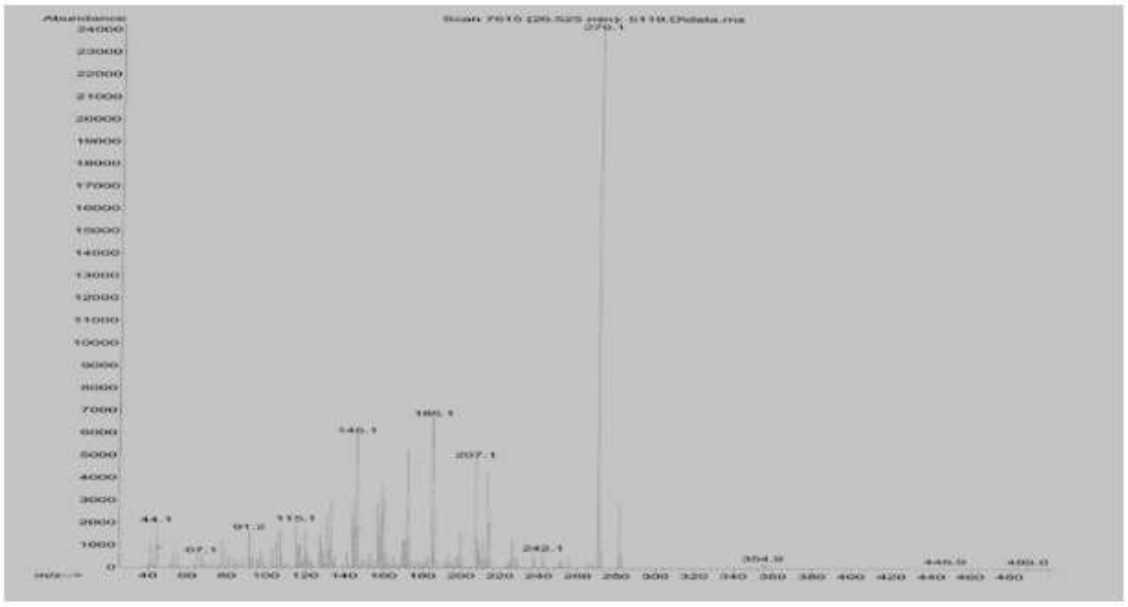
7.2.2 GC-MS results

A different range of chemicals were obtained from the GC-MS separation. Below are the chromatograms for standard hormones, and the urine samples. One target pharmaceutical, estrone, was detected in a very low quality, this could be because the concentration of these estrogens are very low in the urine samples and they need derivation; the fact that the used method was developed to separate anabolic steroids and not the estrogenic ones; the treatment of the sample may not have been able to make the estrogens in the urine volatile enough.

Below are represented the chromatograms of standard solutions and urine samples. The chromatograms for individual substances are represented in appendix 2. The database used to compare the chromatograms was Wiley7n.1.

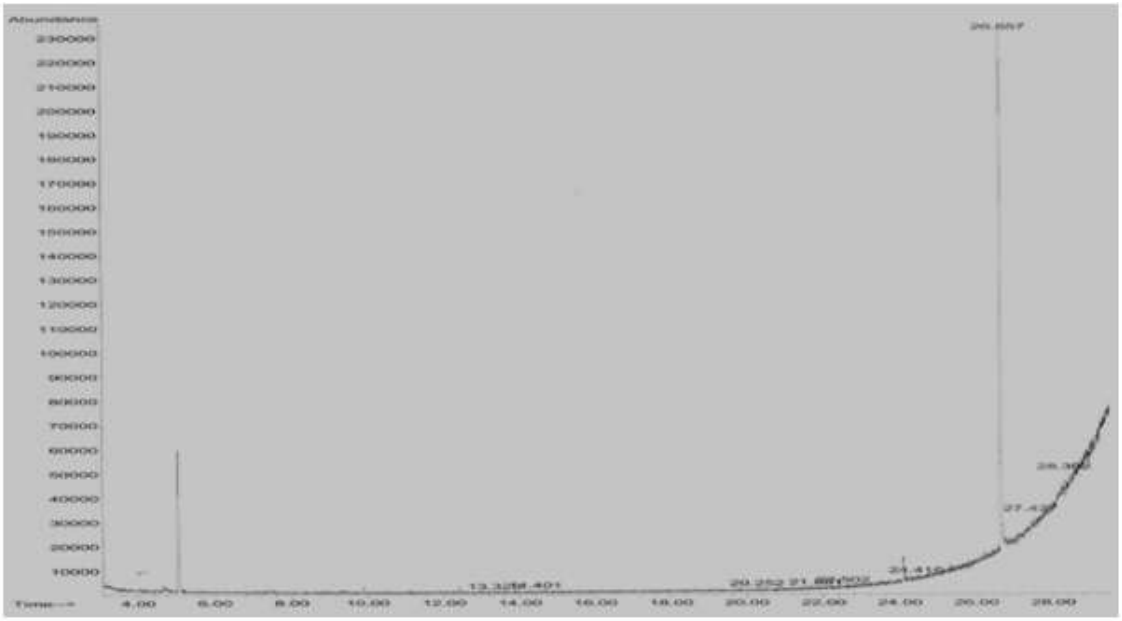


a

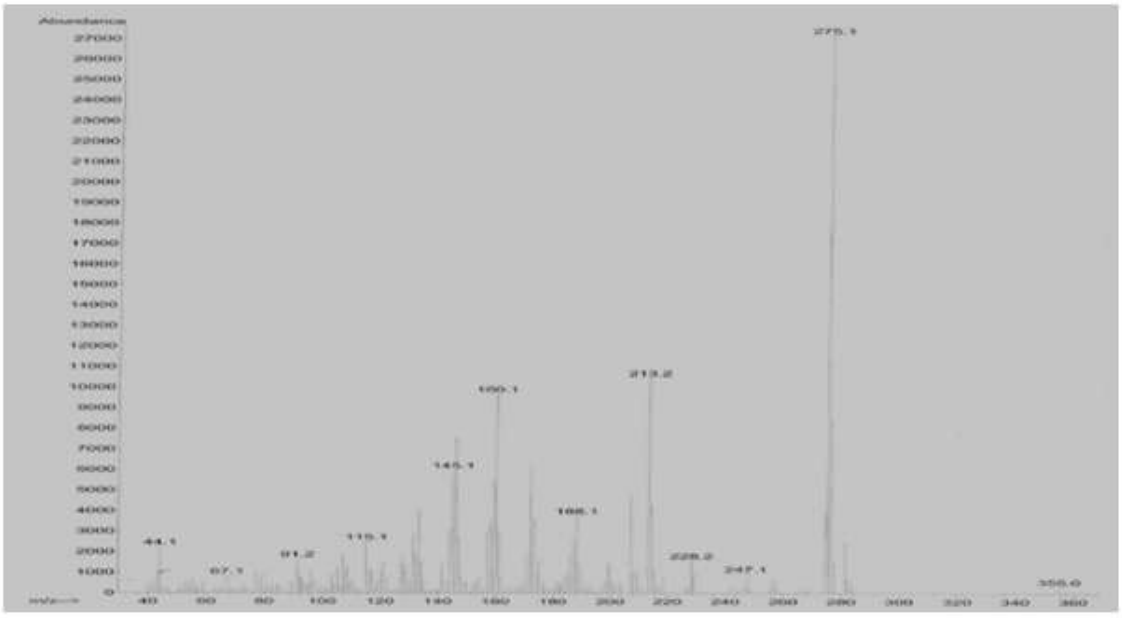


b

Chromatogram 4: Image a represents the chromatogram of estrone standard solution with retention time 26,527 minutes. Image b represents the mass spectrum of estrone with M 270.1.

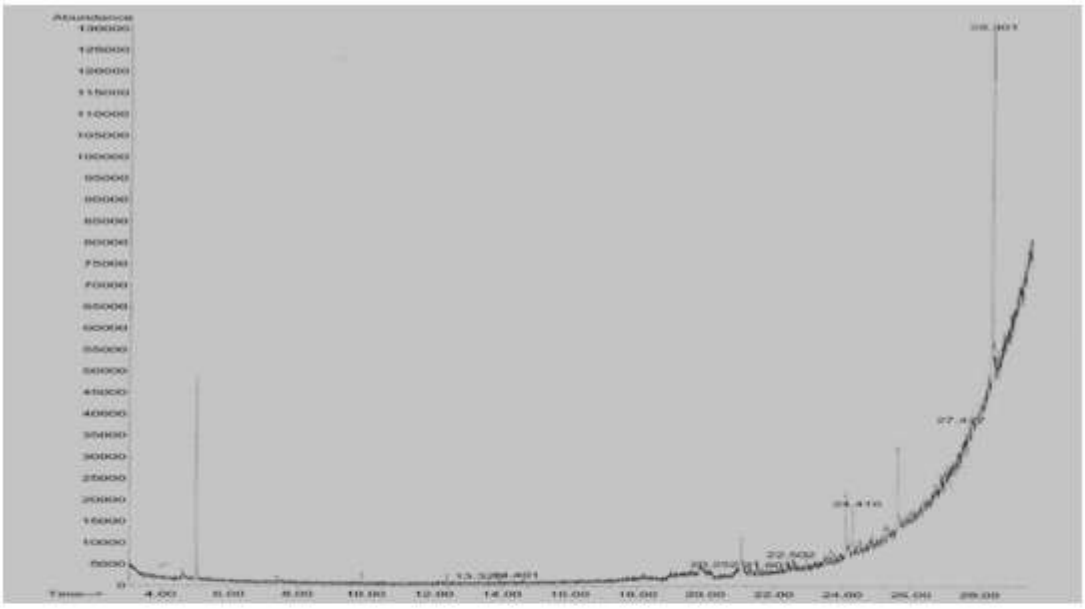


a

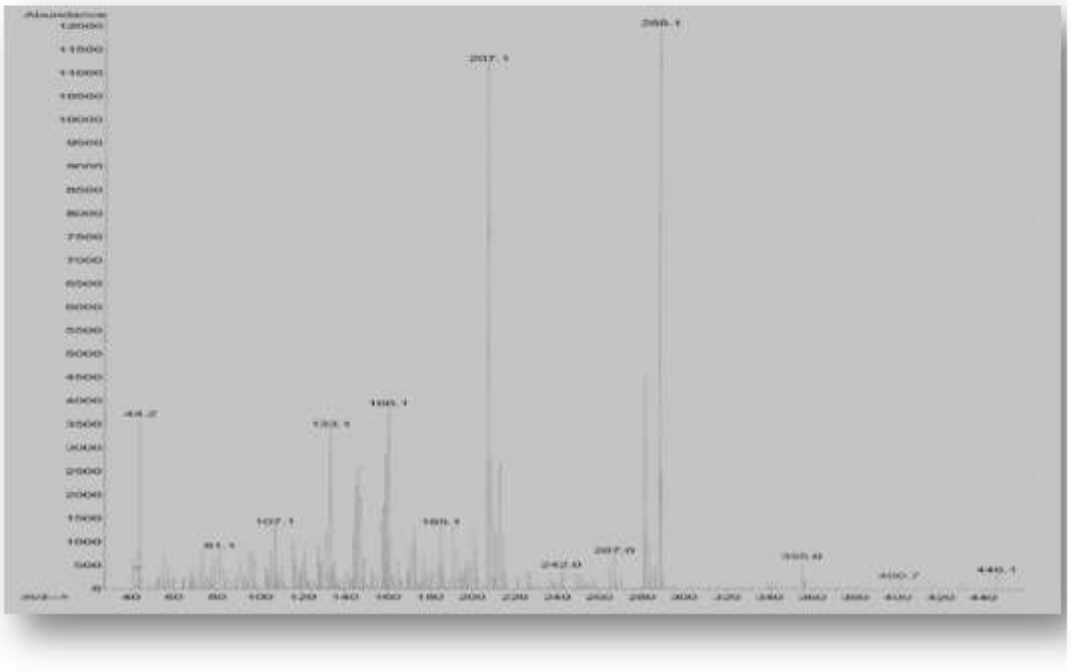


b

Chromatogram 5 Image a represents the chromatogram of 17β-estradiol standard solution with retention time 26,657 minutes. Image b represents the mass spectrum of 17β-estradiol with M 275.1.

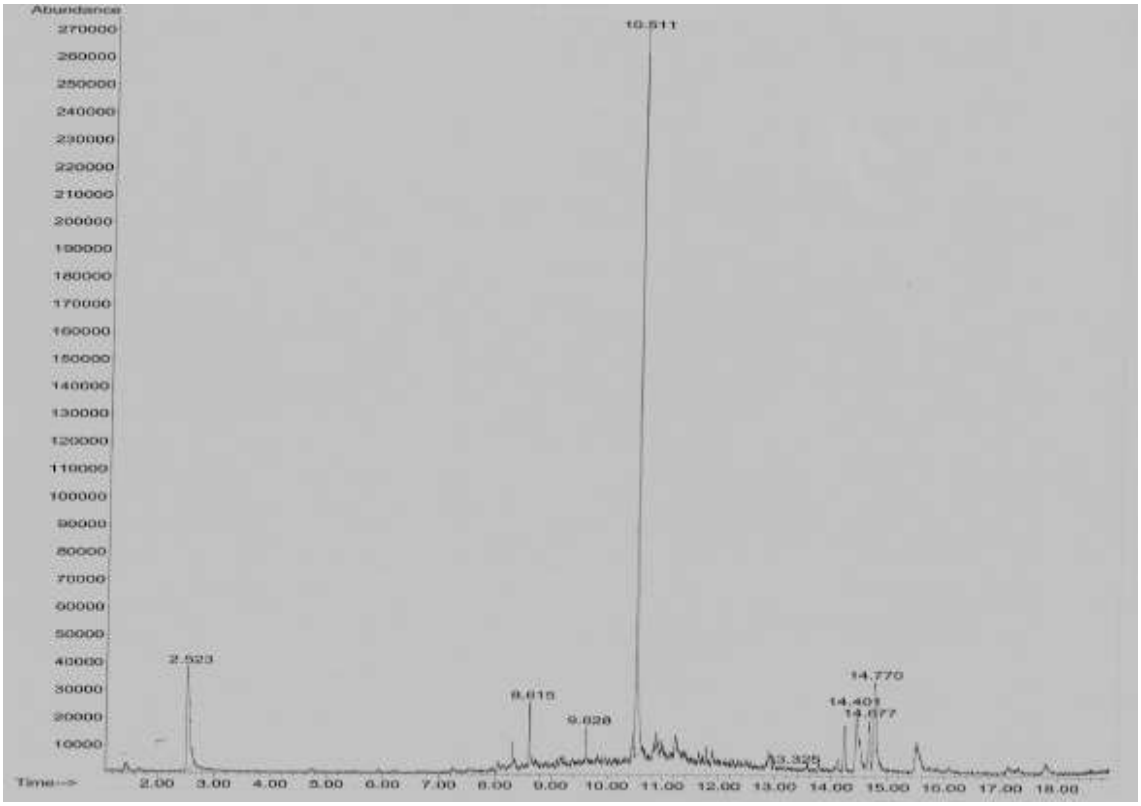


a

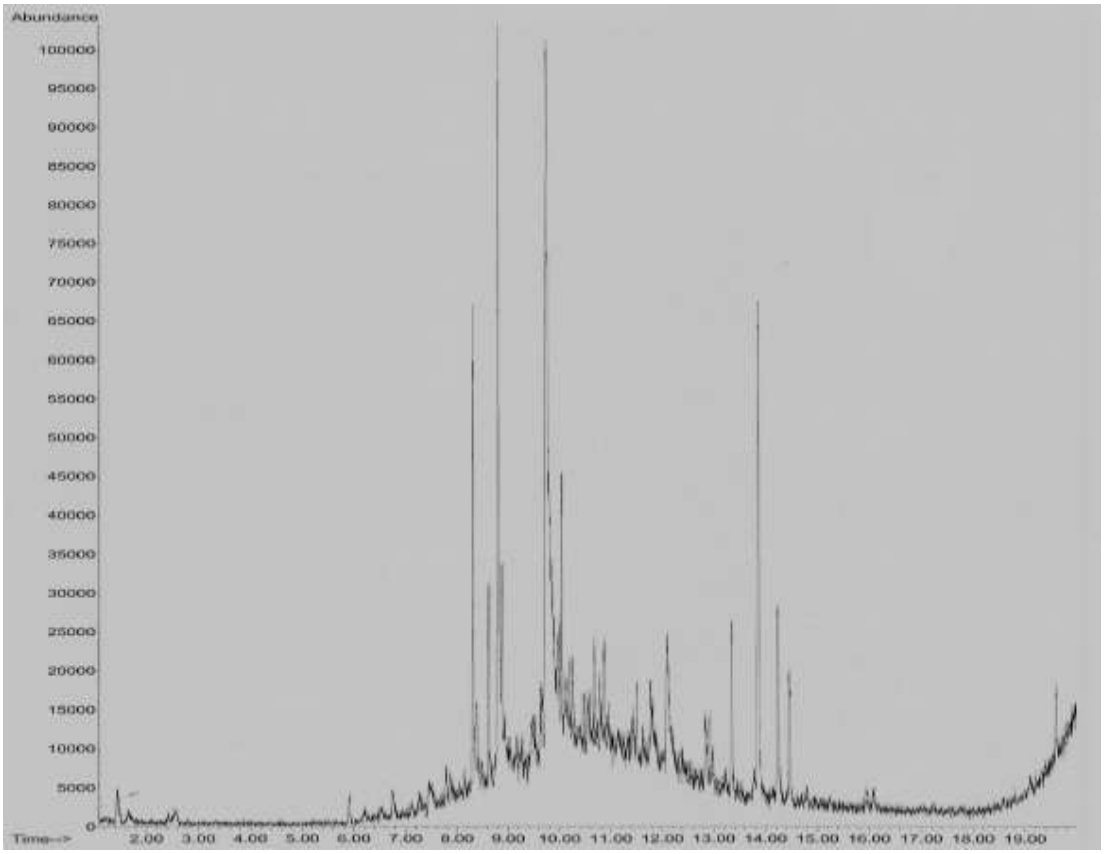


b

Chromatogram 6 Image a represents the chromatogram of estriol standard solution with retention time 28,301 minutes. Image b represents the mass spectrum of estriol with M 288.1.



Chromatogram 7: urine somple from one individual, the substances detected in the urine were caffeine (10,511 min,) 3-hydroxy androstan-17-one(14,667), bis (2-ethylhexyl) phtalate (14,426) and e-321 (8.615)



Chromatogram 8: Urine sample from stored urine, the substances detected were Epiandestrone(14,499 minutes) Di-(2-ethyl) phtalate (14, 466) BHT-acid (8,308) 4-toluenesulfonamide (14,737)

8 DISCUSSION

8.1 Nutrients

Human urine contains valuable nutrients that can be used in agriculture. The concentration of these nutrients in urine depends on local diet. The conditions of urine storage and transportation also affect the final concentrations of nutrients in urine, specially nitrogen, that can easily be lost in gaseous form.

In previous studies, the concentration of nitrogen in stored urine, varied between 1,795 g/L and 2,610 g/L (Kirchmann &Pettersson, 1995,3). The measured concentration of total nitrogen in this experiment was 1,440 g/L. The low concentrations may be due to losses during storage, dilutions in the urine diverting toilets, and the diet of the persons using the toilets.

The concentrations of phosphorus and magnesium were also lower when compared with other studies but the calcium concentration was higher than other studies, as shown in table 8. All studies have the same pH values, but there is differences on the concentration of the nutrients specially nitrogen. These differences may be due to diet patterns as well as storage conditions.

Table 9 Result of nutrient concentrations from different studies.

Measurements	Study 1[a]	Study 2[b]	Study 3[c]	Study 4[d]
pH	9,1	9,1	9,15	9,06
N	9200	9200	9000	1440
P	540	313	344	130
K	2200	1000	1200	1330
Ca	0	18	13	2,5
Mg	0	11	1,9	0,76

1 [a]Maurer et al.(2006);[b]Jönsson et al. (1997);[c] Institute of Environmental Engineering (ISA), ([a,b,c] according to Gethke, Herbes &Pinnekamp); [d] results of this paper

Despite the fact that the concentrations of nutrients were lower when compared to other studies, the studied urine was used with success to grow *Lactuca sativa* in greenhause conditions. The concentration of magnesium on the leaves of *Lactuca sativa* irrigated with urine was higher than in leaves irrigated with artificial fetilizer (Mburu, 2012,33).

The study of microorganisms was not conducted. However the concentrations of nitrogen were over 1 g/L and the pH was 9, and storage time was over six months. According to Shönning(2001,26), these conditions would reduce the amount of pathogens in urine and the urine could be used to fertilize all kind of crops providing that the consumption would take place at least four weeks after the application of the fertilizer.

8.2 Pharmaceuticals

The aim of this thesis was to determine pharmaceuticals in stored urine, specially female estrogens. During the experiments, female oestrogen, estrone, was identified, as well as a number of chemicals which are suspected to be EDCs.

The standard estrone solution, 1 mg/L, was determined by HPLC, with retention time 5,998 minutes. In stored urine, estrone was identified after 6,003 minutes. After comparing the results with literature, they were accepted to be correct. In different studies, estrone has been identified by high performance liquid chromatography using

different methods, columns and wavelengths. Lunn(2000,128) detected estrone from solutions using Radial-PAK µBondapak C18 column with 6,9 minutes of retention time and 214nmwavelength. In soils, estrone was detected by a 25.0cm X 4.6mm Partisil 10 ODS-3 column. The retention time was of 22 minutes and the wavelength was 220 nm (Colucci, Bork & Topp, 2001).

Metabolites of anabolic steroid testosterone and metabolites of dehydroepiandrosterone were identified. Identified testosterone metabolites are androsterone and 3α-hidroxy-5β-androstane-17-one. Dehydroepiandrosterone metabolites are epiandrosterone and prasterone 3-sulfate. These metabolites were identified without spiking the samples. Using the same method, described in section 6.5.3 , Zhang et al. (2008) were able to identify anabolic steroids,3α-hidroxy-5α-androstane-17-one, methyltestosterone, dihydrotestosterone and androstenedione. These hormones were identified after samples were spiked with 50µg/mL of the anabolic standards solutions.

Other identified substances were bis (2-ethylhexyl) phtalate , butylated hydroxytoluene (E-321), (Di-(2-ethyl) phtalate and 4-toluenesulfonamide. Most of these chemicals are used as plasticizers. However, they may have other applications. For example, p toluene sulphonamide is also used in the production of tolbutamide a sugar regulator medicine. 4-methyl phenol and 2,6-bis(1,1-dimathylethyl)-4-methyl also known as E321 and metabolite, are also used in the food industry as food preservative and flavouring respectively.

The presence of this many plasticizer agents in stored urine may be because they were excreted in urine and did not degrade, or because urine was stored in polypropylene plastic containers and some of the containers components dissolved. For example, one of the antioxidants used to protect the structure of polypropylene plastics is butylated hydroxytoluene, and it has been identified in mineralized drinking water (Tombesi & Freije, 2002).

The objectives of this papers was to evaluate the safety of urine reuse in crop production. The safety was evaluated by identifying the presence of pharmaceuticals in urine specially female estrogens. Even though estrone has been identified, this does not mean that it represents risks to the human health. The quantification of hormones and their effects on plants irrigated with estrone containing urine needs to be further investigated.

The other identified chemicals, such as 4-methyl phenol, are suspected to be endocrine disruptors. However, these substances are also used to produce plastics and the urine may have been contaminated during the storage.

In the future, this work can be continued in different areas. In the area of nutrients, sulphur, chlorine, sodium and Chemical Oxygen Demand could be determined. The inactivation of microorganisms could also be studied.

In terms of pharmaceuticals, the used methods need validation. The hormones could also be quantified and their biodegradation can also be studied. Another existent possibility to continue this work would be to analyse urine using the standard addition method.

For future reference, some steps need to be taken to reduce interferences during analysis. For example, glass wear needs to be pre-treated with organic solvents and properly stored. The instruments must be calibrated. Urine samples to be analyzed and standards solutions need to be stored in dark glass containers at temperatures equal or lower than 4°C. If dark glass wear is not available, samples and standards can be stored in transparent glass containers covered with foil.

9 CONCLUSION

Human urine contains valuable nutrients which can be used in agriculture. The concentration of these nutrients in urine depends on local diet. The conditions of urine storage and transportation also affect the final concentration of nutrients in urine, specially nitrogen, that can easily be lost in gaseous form.

The small concentration of nitrogen in the urine does not present any impediment to the use urine as fertilizer. It only means that for crops which require larger amounts of nitrogen, bigger quantities of urine will be required.

Estrone and other endocrine disruptor chemicals were identified in this paper. However, further investigation is required to determine the effects of EDCs on crops irrigated with estrogens containing urine, and the effects to people and animals consuming these crops.

REFERENCES

Adams, C. 2009. Pharmaceuticals. In: A. Bhandari et al., 2009. *Contaminants of emerging environmental concern*. Virginia: American society of civil engineers.

Andersen, H., Siegrist, H., Halling-Sørensen, B., and. Terne, T. 2003. *Fate of Estrogens in a Municipal Sewage Treatment Plant*. Environmental science and technology.37(18):4021-4026. Read 15 May 2012. Available at: http://pubs.acs.org

Akinyemi, O. M. 2007. *Organic and Conventional Systems*. Science publishers. [ebook] Read: April 2012 Available at: http://www.ebrary.com/corp/

Brown, G. 2006. *The effects of estrogens on the growth and tuberization of potato plants (Solanum tubersum ev. "iwa") grown in liquid tissue culture media.* [online] Read: 11 November 2011
Available at: http://ir.canterbury.ac.nz/bitstream/10092/1376/1/thesis_fulltext.pdf

Brunzel,R.2006. *Biochemistry laboratory: Modern theory and techniques*.2nd ed. New Jersey: Pearson.

Centers for disease Control and Prevention,2003. *Leptospirosis* (pdf). Read 11.05.2012. Available at: http://www.cdc.gov/leptospirosis/pdf/fact-sheet.pdf

Centers for disease Control and Prevention,2005. *Human tuberculosis caused by Mycobacterium bovis* New York city, 2001-2004. Read 11.05.2012. Available at: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5424a4.htm

Centers for disease Control and Prevention,2010. *Cytomegalovirus* (CMV) and congenital CMV infection. Read 11.05.2012. Available: http://www.cdc.gov/cmv/index.html

Centers for disease Control and Prevention,2012a. *E.Coli*. Read 11.05.2012. Available at: http://www.cdc.gov/ecoli/

Centers for disease Control and Prevention,2012b. *Microsporidiosis.* Read 11.05.2012 Available at: http://dpd.cdc.gov/dpdx/HTML/Microsporidiosis.htm

Centers for disease Control and Prevention,2012c. *Tuberculosis (TB).* Read 11.05.2012. Available at: http://www.cdc.gov/tb/topic/basics/default.htm

Chijide V. 2012. *Microsporidiosis.* 11.01.2012 Read 11.05.2012. Available at: http://emedicine.medscape.com/article/221631-overview

Colucci, M.S.; Bork, H.; Topp, E. 2001. Persistence of estrogenic hormones in agricultural soils: I 17β-estradiol and estrone. Journal environmental Quality.30:2070:2076. Read 20.04.2012.
Available at: https://www.agronomy.org/publications/jeq

Colucci, M.S.; Topp, E. 2001. Persistence of estrogenic hormones in agricultural soils: II 17α-ethynylestradiol. Journal environmental Quality.30:2077:2080. Read 20.04.2012.
Available at: https://www.agronomy.org/publications/jeq

Dean, J., Jones, A., Holmes, D., Reed, R., Weyers, J. and Jones, A. 2002. *Practical skills in chemistry*. Harlow: Pearson education.

Easmon, C. 2009. *Healthy living. Typhoid fever and paratyphoid fever*. 20.08.2009. Read. 15.05.2012. Available at: http://www.netdoctor.co.uk/travel/diseases/typhoid.htm

Encyclopedia Britannica. *Pharmaceutical*. Read: 10.8.2012. Available at: http://www.britannica.com

Freire López, Alberto, 2012. *Scale model testing of leachate treatment with willow stack tower and ebb-flow systems* (pdf). Tampere:Tampere University of applied sciences.

Gethke, K., Herbst, H., Pinnekamp, J. Human urine decomposition processes and nutrient recovery. Institute of environmental engineering (ISA). Read 24.11.2012. Available at: http://www.researchgate.net/

Gimeno, S., Komen, H., Jobling, S., Sumpter J., Bowmer, T. 1998. *Demasculinisation of sexually mature male common carp, Cyprinus carpio, exposed to 4-tert-pentylphenol during spermatogenesis*. Aquatic Toxicology, Volume 43, Issues 2–3, 1 October 1998, Pages 93-109

International Agency for Research on Cancer. *Schistosoma Haematobium.* (pdf). IARC Monographs 100b. Read 23.05.2012. Available at: http://monographs.iarc.fr/ENG/Monographs/vol100B/mono100B-14.pdf

Jobling, S. Williams, R. Johnson, A. TAylor, A. Gross-Sorokin, M. Nolan, M. Tyler, C. van Aerle, R. Santos, E. and Brigthy, G. 2005. *Predicted Exposures to Steroid Estrogens in U.K. Rivers Correlate with Widespread Sexual Disruption in Wild Fish Populations.* Environmental Health Perspectives [online] Read: 14 April 2012
Available at: http://ehp03.niehs.nih.gov/article/info:doi/10.1289/ehp.8050

Keil, F. 2008. Institute for social-ecological research(ISOE)GmbH. *Pharmaceuticals for human use: options of action for reducing the contamination of water bodies.* Read 30.10.2012. Available at: http://www.start-project.de

Kirchmann, H., Pettersson,S., 1995. *Human urine chemical composition and chemical efficiency*. Fertilizer research 1994/1995, Volume 40, 149-154. Read 24.11.2012. Available at: http://link.springer.com

Kitson, F., Larsen, B. McEwen, C. 1996. *Gas Chromatography and Mass Spectrometry: A practical guide.* Academic Press. [ebook] Read: 5 October 2012 Available at: http://site.ebrary.com.elib.tamk.fi

Knudsen, M., Halberg, N., Olesen, J., Byrne, J., Iyer, V. and Toly, N. 2006. Global trends in agriculture and food systems. In: R. Mosier at al., 2006. N. Halberg. Global development of organic agriculture: challenges and prospects. [ebook] Read: 20 April 2012. Available at: http://www.ebrary.com/corp/

Kümmer, K. 2004. Pharmaceuticals in the environment scope of the book and introduction. In: *Pharmaceuticals in the environment, sources, fate, effects and risks*. New York:Springer.

Leptospirosis Information Center, 2011. Overview of the leptospira bacterium itself. Read: 5 April 2012. Available at: http://www.leptospirosis.org/

Lunn, G. 2000. *HPLC methods for pharmaceutical analysis., volume 3: E-O.* New York. John Wiley and sons.

Limpiyakorn, T., Homklin, S., Ong, S. 2009. Hormones. In: A. Bhandari et al., 2009. *Contaminants of emerging environmental concern*. Virginia: American society of civil engineers.

Mao, L., Sun, C., Zhang, H., Li, X. and Wu, D. 2004. *Determination of environmental estrogens in human urine by high performanve liquid chromatography after fluorescent derivatization with p-nitrobenzyl chloride.* Analytica Chimica acta (2004) 241-246.

Mathews, C. Van Holde, K., Ahern, K. 2000. *Biochemistry*. 3rd ed. San Francisco: Addison Wesley Longman. [ebook]

Miège, C., Gabet, V., Coquery, M., Karolak, S., Jugan, M-L., Oziol, Y., Levi, Y. and Chevreuil, M. 2009. *Evaluation of estrogenic disrupting potency in aquatic environments and urban wastewaters by combining chemical and biological analysis*. TrAc Trends in Anaöytical Chemistry, Volume 28, issue 2, February 2009.186-195.

McMaster, C., 2007, HPLC: a practical user's guide. 2nd ed. Hoboken, NJ: Wiley-interscience.

McMaster, M., McMaster, C. 1998. *GC/MS A practical user's guide*. New York: Wiley-VHC.

Metcalfe, C., Miao, X-S., Hua, W., Letcher, R. and Servos, M. 2004. Pharmaceuticals in the Canadian environment. In: K. Kümmer, 2009. *Pharmaceuticals in the environment, sources, fate, effects and risks*. New York:Springer.

Mosier, A., Syers, J., Freney, J. 2004. *Agriculture and nitrogen cycle: Assessing the impacts of fertilizer use on food production and the environment*. Island Press. Read: 1 April 2012 Available at: http://site.ebrary.com.elib.tamk.fi

Mburu, C.,2012. *Urine used as a fertilizer for lettuce grown in greenhouse conditions*. Tampere:Tampere University of applied sciences. Read 25.11.2012. Available at: http://publications.theseus.fi/

Norman, A., Litwack, G., 1997, Hormones 2nd ed. San Diego: Academic press.

Pacific Institute, 2010. *World water quality facts and statistics*. Released 22.03.2010. Read 24.05.2012. Available at: http://www.pacinst.org/reports/water_quality/water_quality_facts_and_stats.pdf

Panter, G. H., Thompson, R. S., Sumpter, J.P., 1997. *Adverse reproductive effects in male fathead minnows (Pimephales promelas) exposed to environmentally relevant concentrations of the natural oestrogens, oestradiol and oestrone.* Aquatic Toxicology, Volume 42, Issue 4, August 1998, Pages 243-253

Schulte-Oehlmann u. et al., 2004, Effects of ethinylestradiol and methyltestosterone in Prosobranch snails. In:K. Kümmer, 2004. *Pharmaceuticals in the environment, sources, fate, effects and risks*. New York:Springer

Schönning, C. 2001. *Urine diversion: hygienic risks and microbial guidelines for reuse*. [online] Read: 1 January 2012 Available at http://www.who.int/water_sanitation_health/wastewater/urineguidelines.pdf

Schönning, C., Stenström, A. 2004. *Guidelines for the safe use of urine and faeces in Ecological sanitation Systems*.pdf. Stockholm: EcoSanRes . Read: 23.05.2012. http://www.ecosanres.org/pdf_files/ESR_Publications_2004/ESR1web.pdf

Shore, L., Kapulnik, Y., Gurevich, M., Wininger, S. Badamy, H. and Shemesh M. 1994. *Induction of phytoestrogen production in Medicago sativa leaves by irrigation with sewage water*. Environmental and Experimental Botany, Volume 35, Issue 3, July 1995, Pages 363-369

Somani, L. 2007. Bio-fertilizers: a supplemental source of plant nutrients in IPNS. In: M.K., Gupta,2007. *Handbook of organic farming and bio-fertilizers*. ABD publishers. [ebook] Read: 1 April 2012. Available: http://www.ebrary.com

Steinfeld, C. 2004. *Liquid gold. The lore and logic of using urine to grow plants.* United States: Vermont. Green Frigate books.

The Leptospirosis Information Center, 2011. *Life Cycle of pathogenics leptospires.* Read 22.05.2012. Available at: http://www.leptospirosis.org/topic.php?t=27

Tombesi, N., Freije, H, 2002. *Application of solid-phase microextraction combined with gas chromatography-mass spectrometry to the determination of butylated hydroxytoluene in bottled drinking water*. Journal of chromatography A, 2002, 179-183. Read 25.11,2012. Available at: http://www.sciencedirect.com

Tomšíková H., Aufartová, J. Solich, P., Sosa-Ferrera, Z. Santana-Rodríguez, J. and Nováková, L., 2012. *High-sensitivity analysis of female steroid hormones in environmental samples* (pdf). Read 18.05.2012. Available at: http://www.sciencedirect.com/

Weckman, A. 2001.*Ihmisten ulosteet lannoitteena*. Helsinki: työtehoseuran monisteita1/2000(75).

Ying, G-G., Kookana, R. and Ru, Y-J. 2002. *Occurrence and fate of hormone steroids in the environment* (pdf). Environment International 28 (2002) 545 – 551. 6.09.2002. Read 18.05.2012
Available at :http://www.lu.lv/ecotox/publikacijas-3-kursa-studentiem/Steroid_hormones_EI.pdf

Zavod, R. M., 2008. Women's health. In: T. LEmke at al. Foye's principles of medicinal chemistry.[ebook] Read 27.10.2012. Available at: http://books.google.fi/

Zhang, Z., Duan, H., zhang, L., Chen, X., Liu, W. and Chen, G. 2009. *Direct determination of anabolic steroids in pig urine by a new SPME-GC-MS method* (pdf). Talanta, volume 78, Issue 3, 15 May 2009, psges 1083-1089. Read 5 May 2012. Available at: http://www.sciencedirect.com/

Zhao, J-L et al., 2010. *Estrogenic activity profiles and risks in surface waters and sediments of Pearl River system in South China assessed by chemical analysis and in vitro bioassay* (pdf). Journal of environmental monitoring, 2011, 13, 813-821. Read 17.11.2012. Available at: http://pubs.rsc.org/en/

APPENDIX

Appendix 1 Physicochemical properties of estrogens

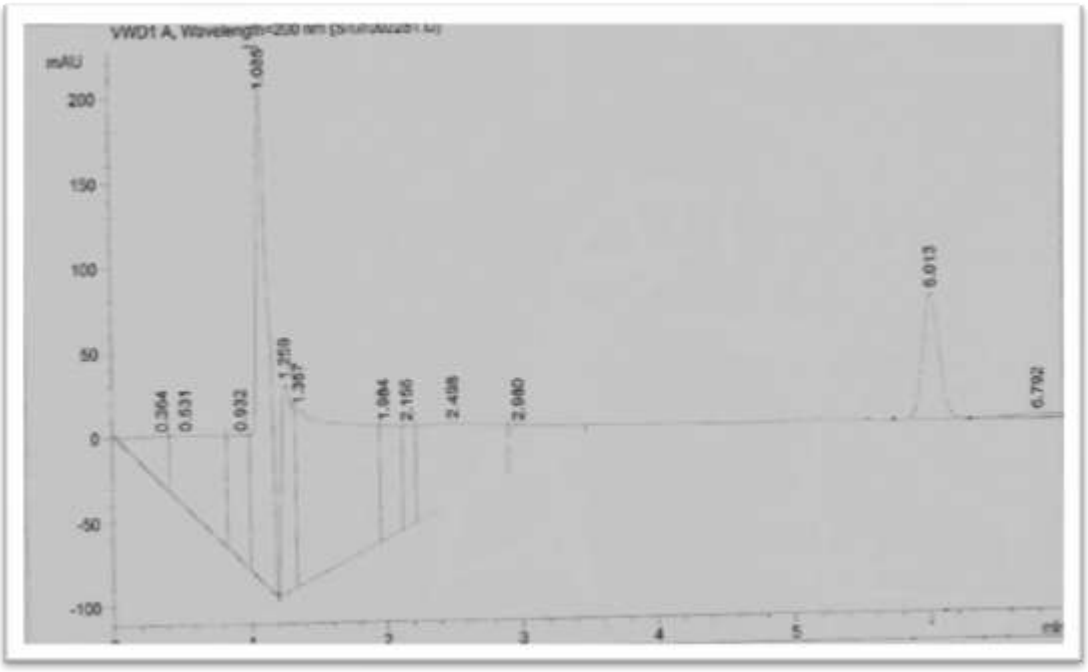
Table 1: Physicochemical properties major estrogens

Hormone	Acronym	structure	Molecular Weight	Solubility in water mg/L	Log KOW*	pKa	Half life (hours)	vapor pressure (mmHg)	KH (atm m3/mole)
Estrone	E1	C18H22O2	270.4	30	3,13	10,3-10,8	19	1,42 X10-7	3.80 X10-10
17β-estradiol	17β-E2 or E2	C18H24O2	272.4	3,6	4,01	10,5-10,7	13	1,26 X10-8	3,64 X10-11
estriol	E3	C18H24O3	288,4	441	2,45	10,4	NA	1,07 x10-9	1,33 X10-12
ethinylestradiol	EE2	C20H24O2	296,4	11,3	3,67	NA	36	2,67 X10-9	7,94 X10-12
Mestranol	MeEEe	C21H26O2	310.4	0,3	4,67	NA		7,5 x10-10	

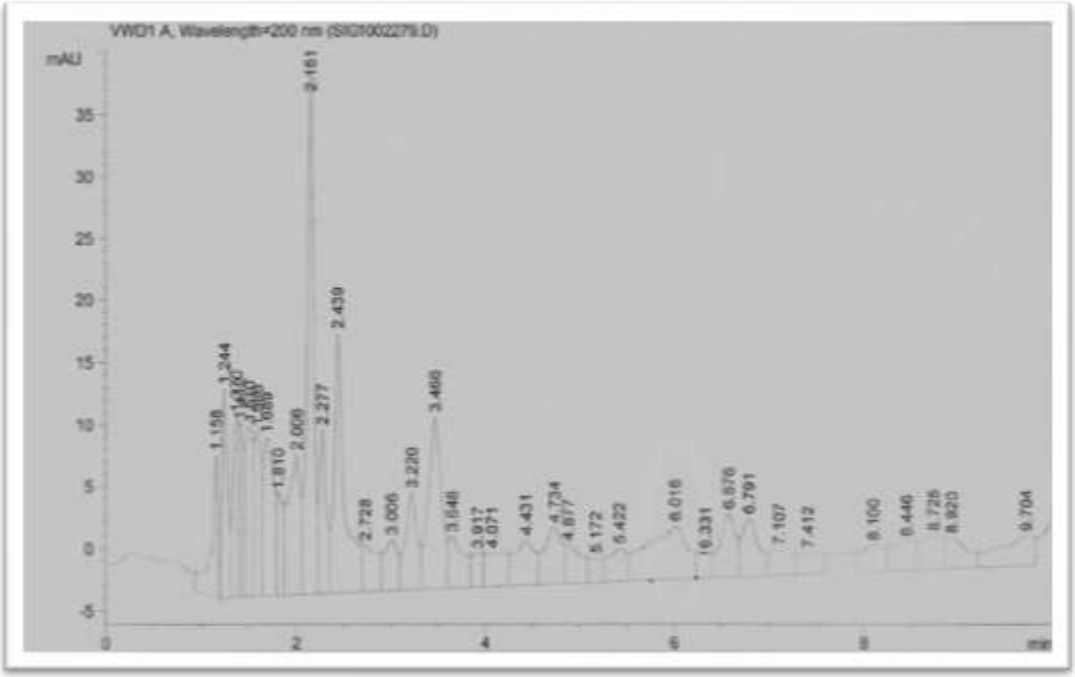
* Log KOW octanol-water partition coeffficient; NA not available. Source: ChemlDplus Lite, 2008 according to Limpiyakorn **Lympiyakorn, Homklin & Ong**;2009, 189 Ying , kokoona & Ru.,2002)

Appendix 2 HPLC chromatograms

Below are the HPLC and GC-MS chromatograms not presented on the main text. The Mobile phase for all the HPLC samples was: A (MeCN: water): C (Na_2HPO_4) v/v 50:50.



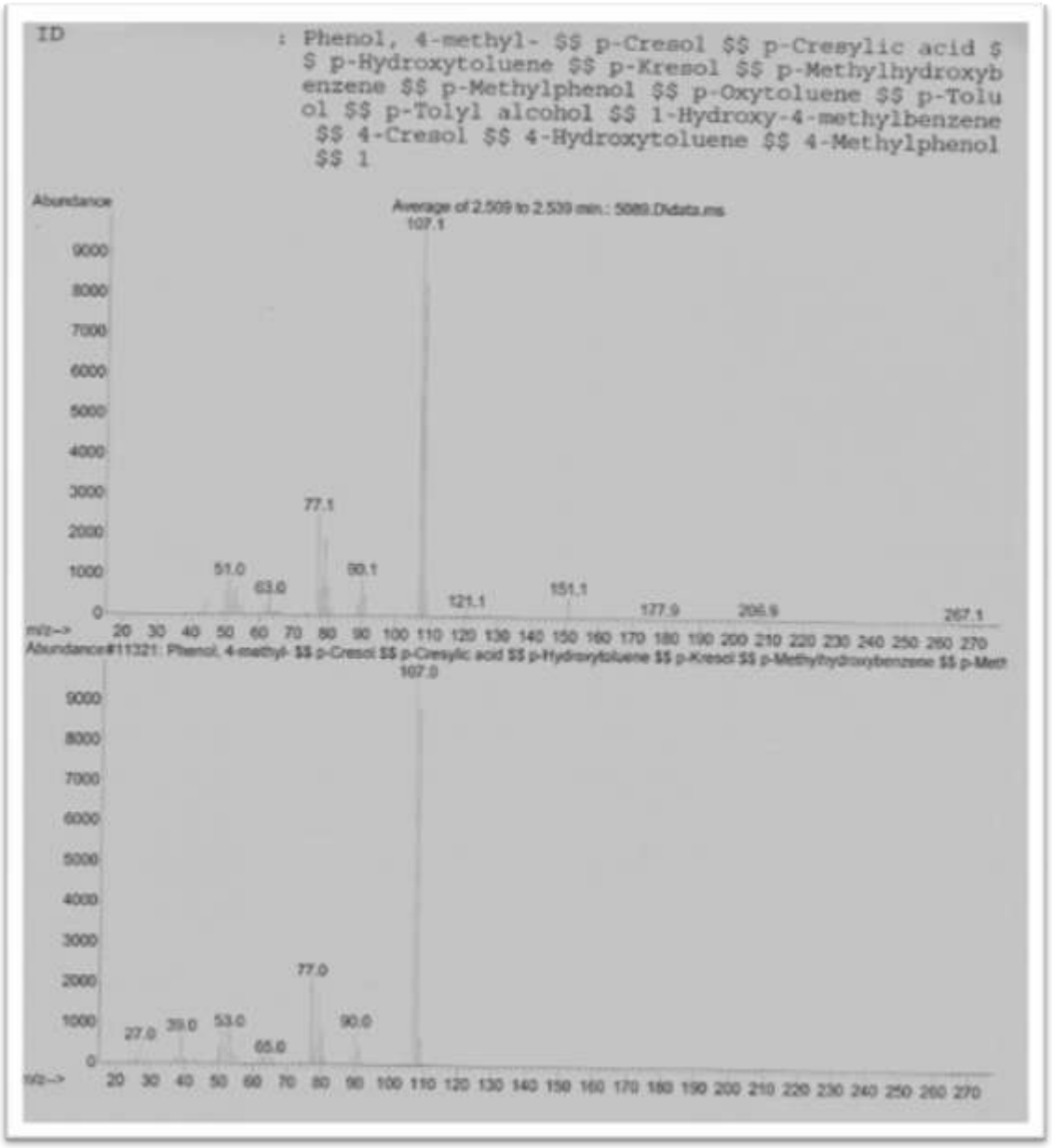
Chromatogram 1: HPLC chromatogram of estrone diluted in acetonitrile without any pretreatment.



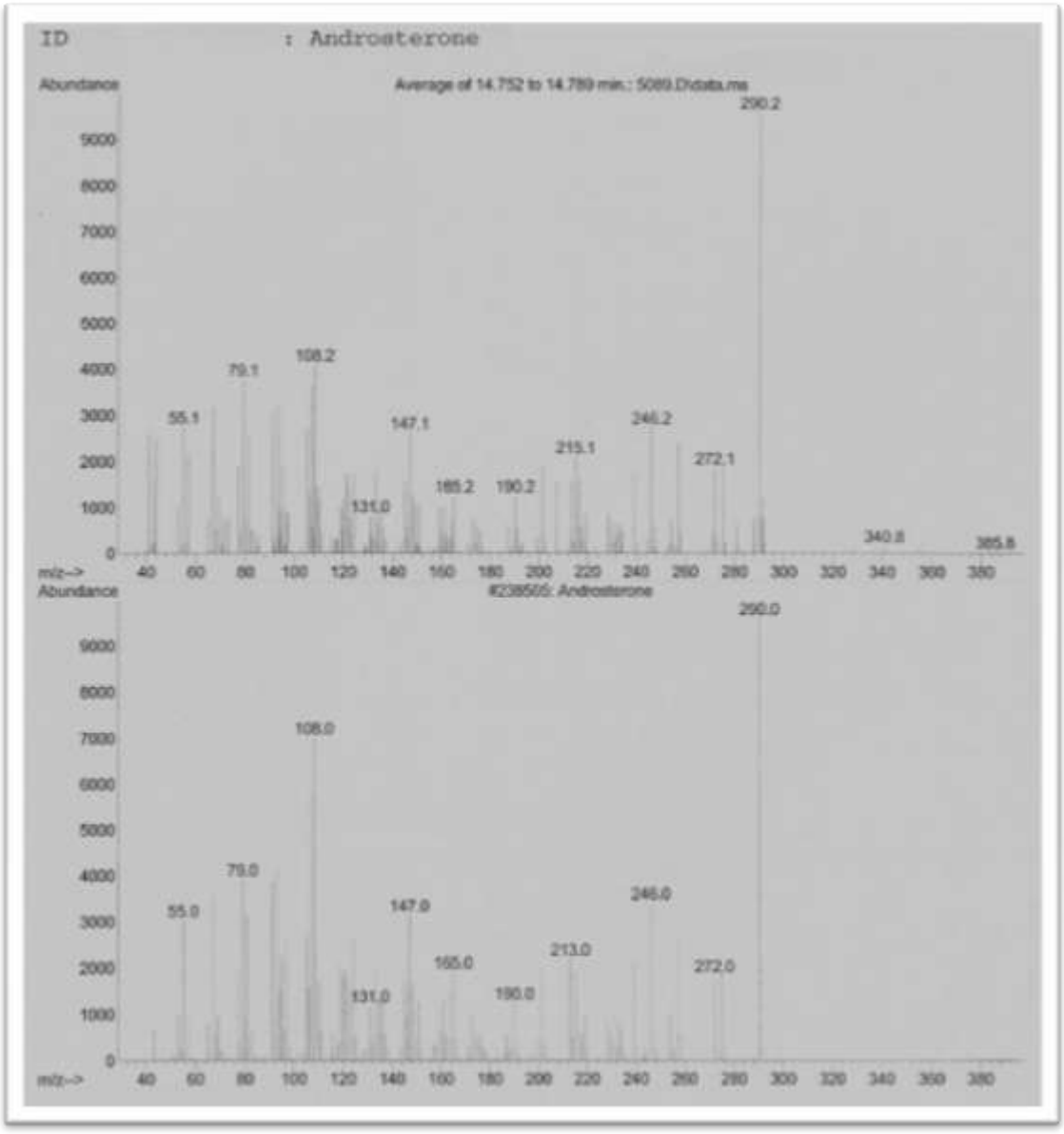
Chromatogram 2: Diluted urine, the organic layer was separated using ToxiTube **A**.

Appendix 3 GCMS chromatograms

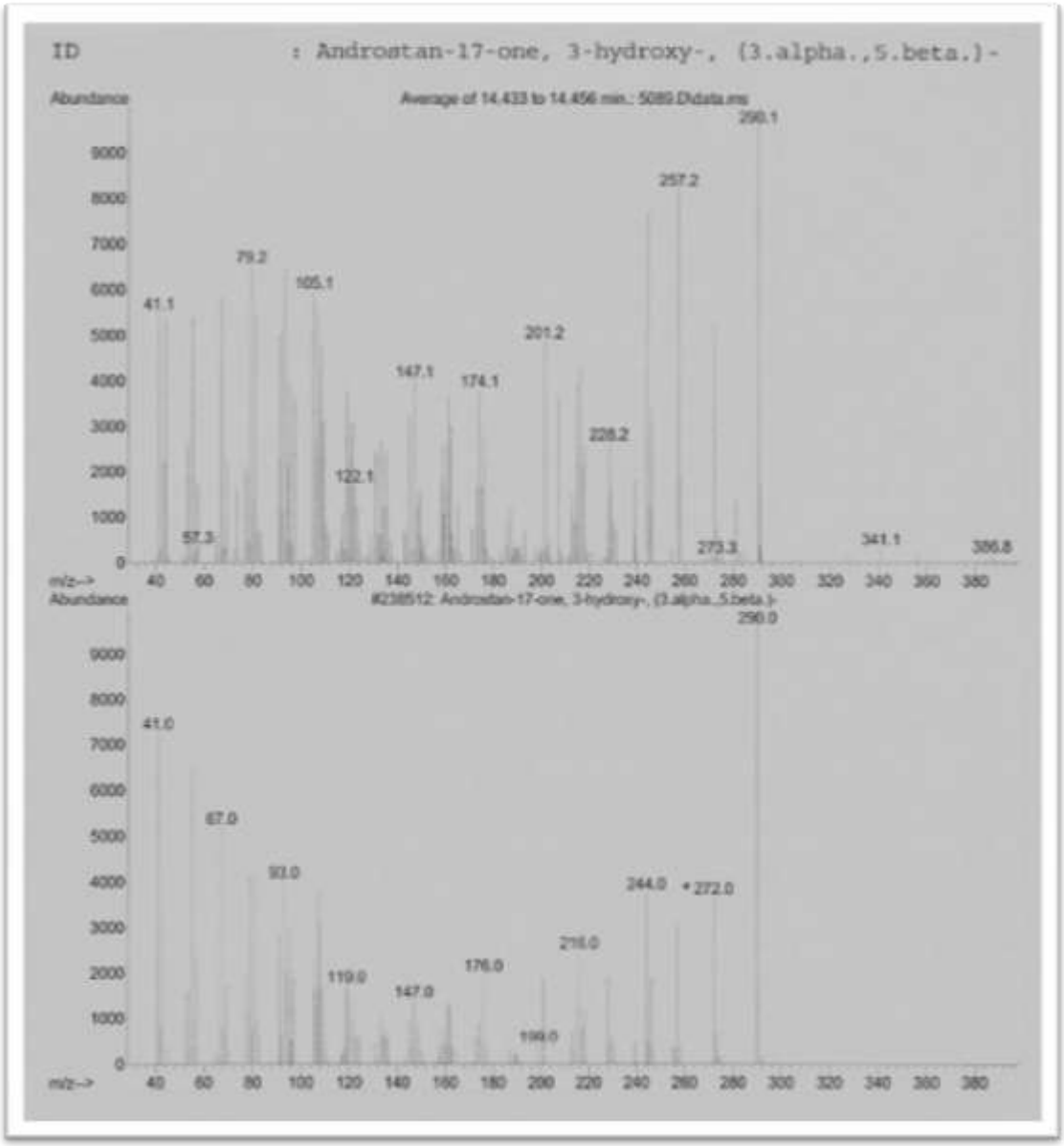
Below are represented the GC-MS chromatograms



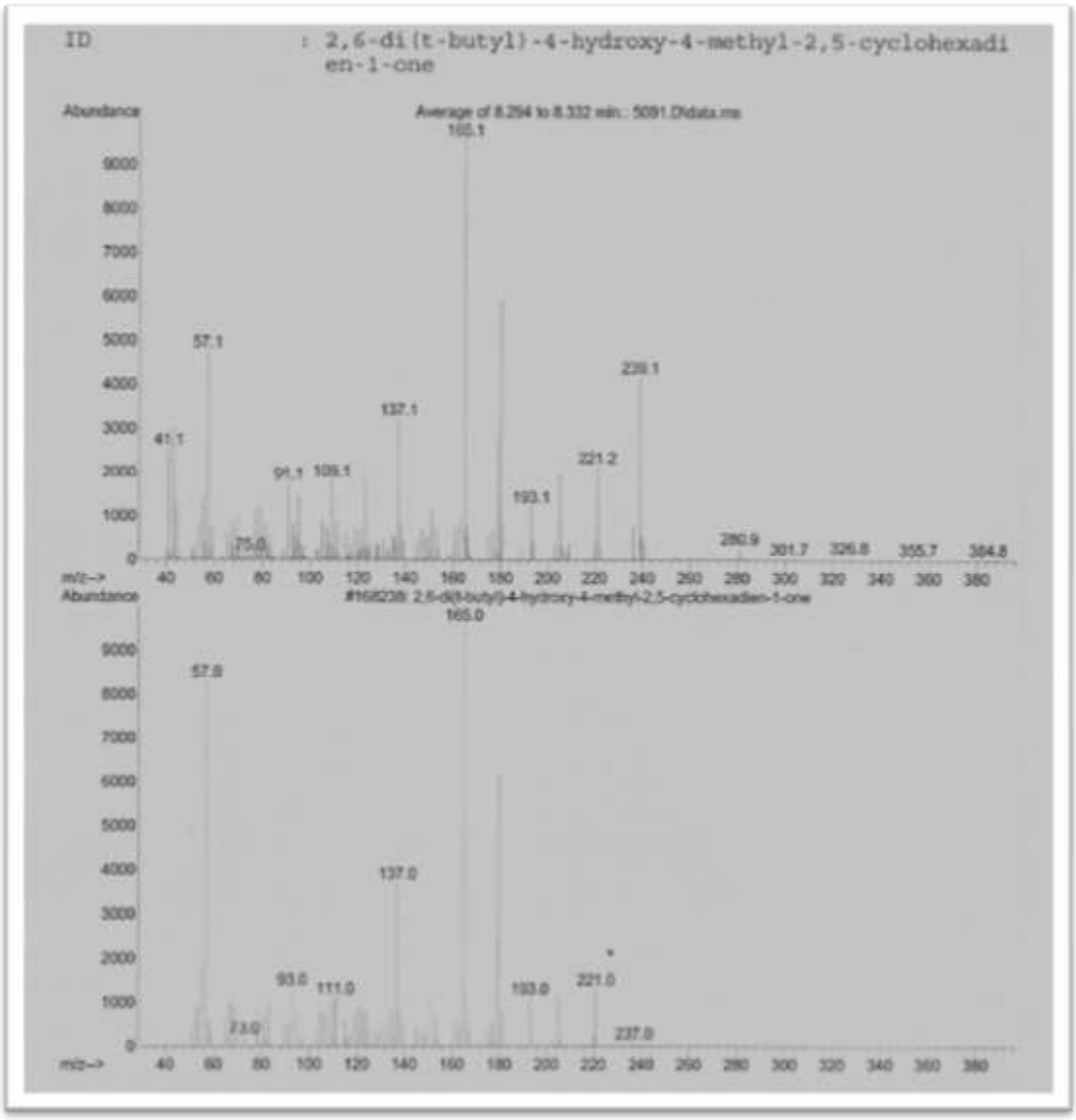
Chromatogram 3: p-cresol the end result of protein breakdown. It also a metabolite on menthofuran a compound found *Mentha pulegium and Hedeoma pulegioides*. It is found in Pennyroyal oil a product used in the flavoring industry due to its mint-smell.



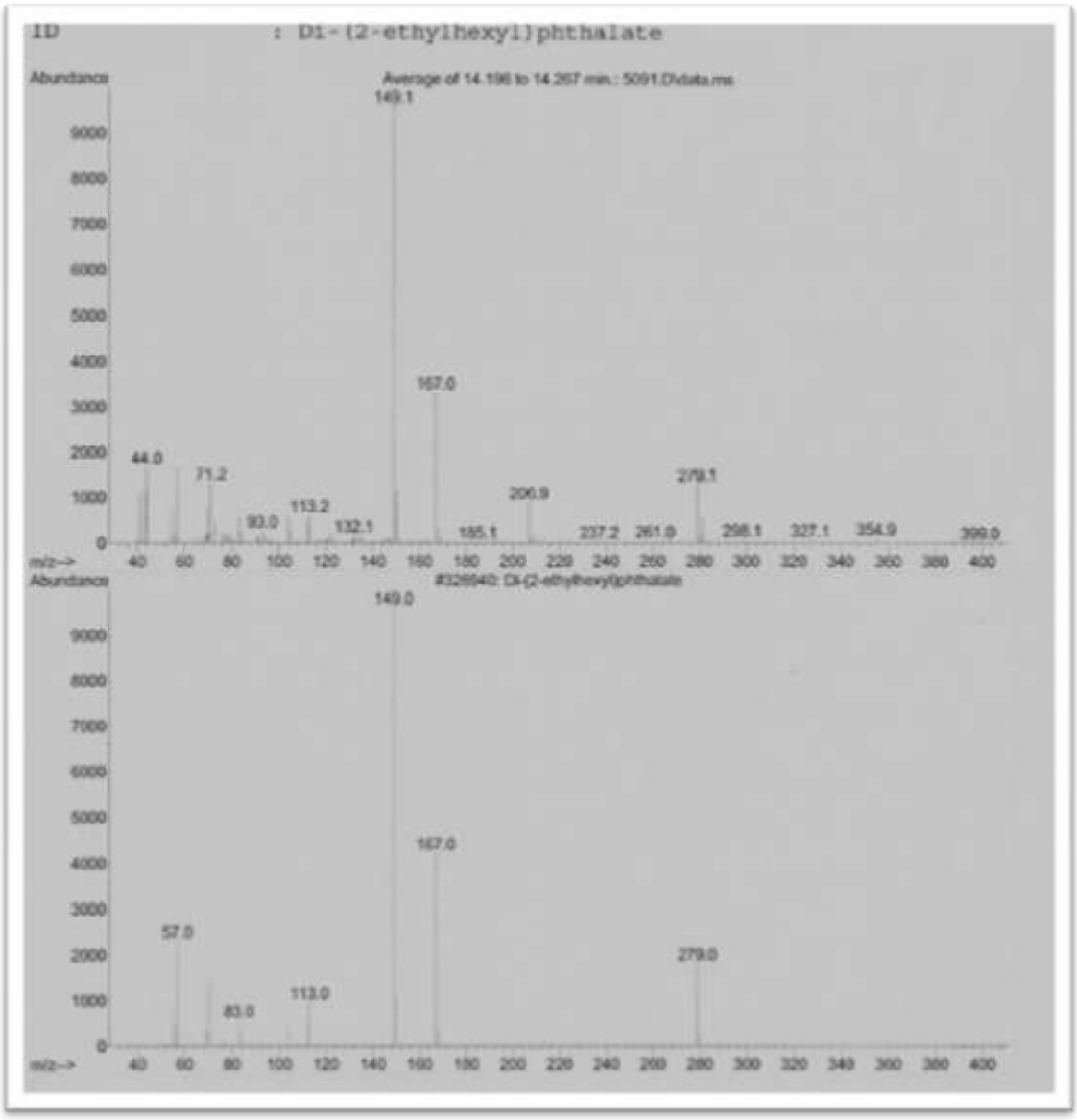
Chromatogram4: Androsterone is an inactive breakdown of testosterone.



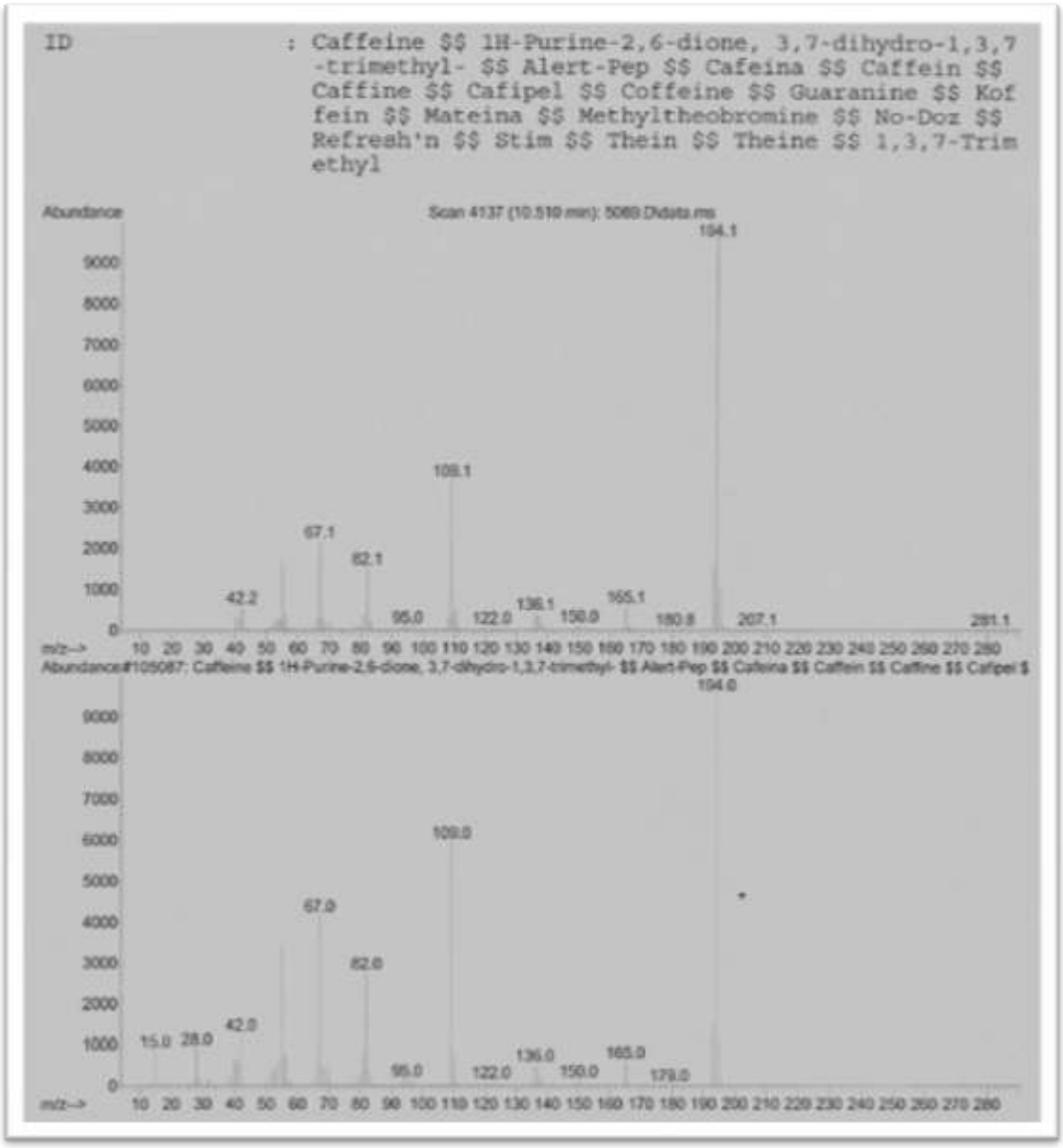
Chromatogram 5: 3-alpha-Hydroxy-5-beta-androstan-17-one is the isomer androsterone.



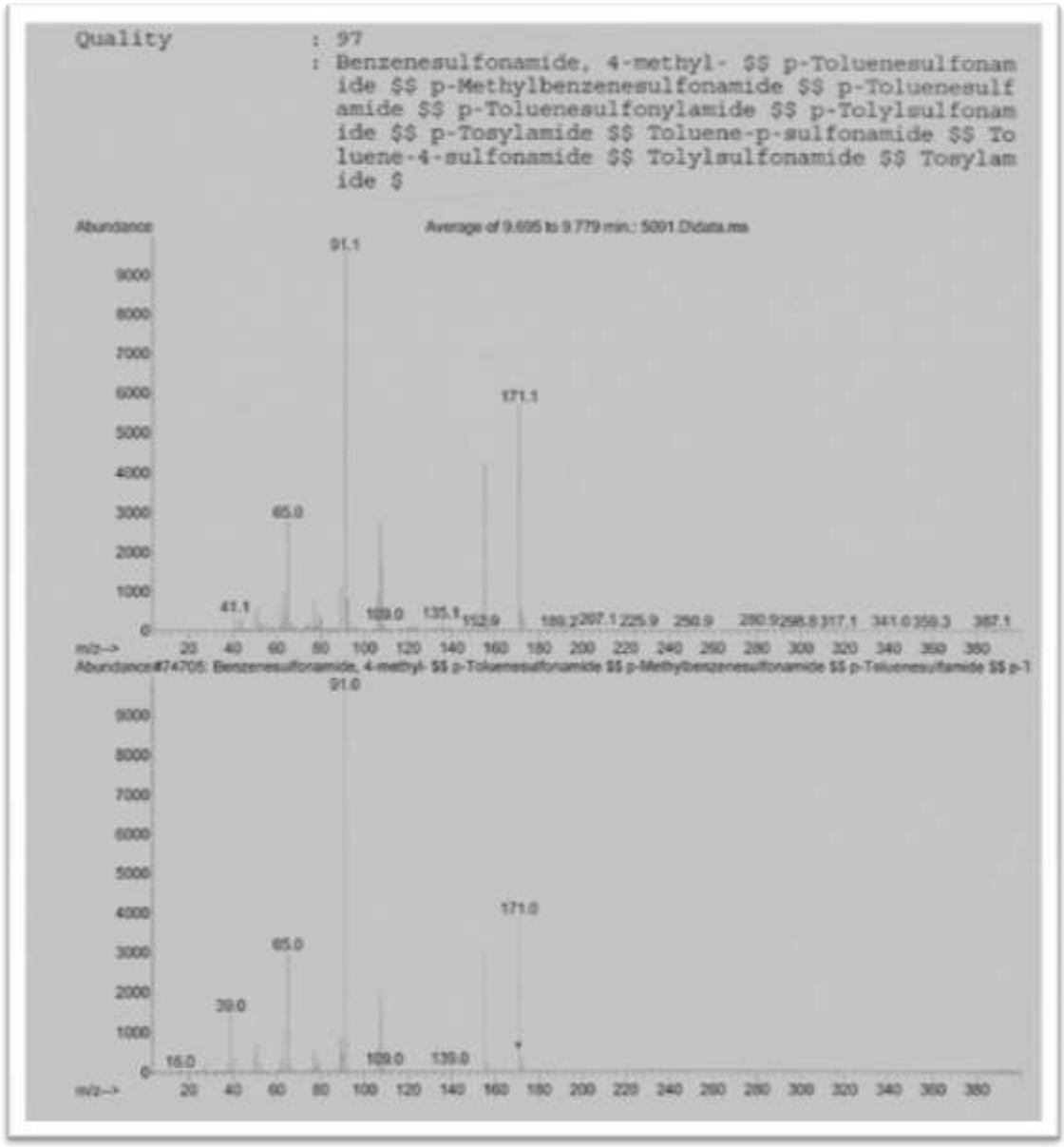
Chromatogram 6: BHT-OH is the metabolite of 2,6-bis(1,1-dimethylethyl)-4-methyl phenol a food antioxidant.



Chromatogram 7: Di-(2-ethylehyl)-phthalate also known as DEHP is a plasticizer.



Chromatogram 8: Caffeine, the most consumed stimulant worldwide.



Chromatogram 2: 4-methyl benzenesulfonamide a plasticizer.

References:

Human metabolome database, 2012. 2,6-Di-tert-butyl-4-hydroxymethylphenol Read 28.10.2012. Available at: http://www.hmdb.ca/

Human metabolome database, 2005. Androsterone. Read 28.10.2012. Available at: http://www.hmdb.ca/

Human metabolome database, 2006. Caffeine. Read 28.10.2012. Available at: http://www.hmdb.ca/

Human metabolome database, 2006. p-Cresol Read 28.10.2012. Available at: http://toxnet.nlm.nih.gov/

Human metabolome database, 2006. p-Cresol Read 28.10.2012. Available at: http://www.hmdb.ca/

Limpiyakorn, T., Homklin, S., Ong, S. 2009. Hormones. In: A. Bhandari et al., 2009. *Contaminants of emerging environmental concern*. Virginia: American society of civil engineers.

United states National Library of Medicine, ToxiNet. *Di-(2-ethylhexyl)-pthalate*. Read 01.11.2012. Available at: http://toxnet.nlm.nih.gov/

United states National Library of Medicine, ToxiNet. *p-toluenesulfonamide*. Read 01.11.2012. Available at: http://toxnet.nlm.nih.gov/